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CANADIAN THESES ON MICROFICHE

THÈSES CANADIENNES SUR MICROFICHE

NAME OF AUTHOR/*NOM DE L'AUTEUR* ROGER GUEVREMONT

TITLE OF THESIS/*TITRE DE LA THÈSE* A Study of the Osterberg-Sarkar-Kruck Method for Evaluating Free Ion Concentrations in Solutions of Complex Equilibria, And a Study of the Complexati Chemistry of Glutathione by this Method.

UNIVERSITY/*UNIVERSITÉ* U. of Alberta

DEGREE FOR WHICH THESIS WAS PRESENTED/ *GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE* Ph. D.

YEAR THIS DEGREE CONFERRED/*ANNÉE D'OBTENTION DE CE GRADE* 1978

NAME OF SUPERVISOR/*NOM DU DIRECTEUR DE THÈSE* D. L. Rabenstein

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A STUDY OF THE OSTERBERG-SARKAR-KRUCK METHOD FOR EVALUATING FREE ION CONCENTRATIONS IN SOLUTIONS OF COMPLEX EQUILIBRIA, AND A STUDY OF THE COMPLEXATION CHEMISTRY OF GLUTATHIONE BY THIS METHOD

by

© ROGER GUEVREMONT

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA
SPRING, 1978

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled A STUDY OF THE OSTERBERG-SARKAR-KRUCK METHOD FOR EVALUATING FREE ION CONCENTRATIONS IN SOLUTIONS OF COMPLEX EQUILIBRIA, AND A STUDY OF THE COMPLEXATION CHEMISTRY OF GLUTATHIONE BY THIS METHOD submitted by ROGER GUEVREMONT in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

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ABSTRACT

The method developed by Osterberg, Sarkar and Kruck for obtaining the concentrations of free ions in solutions of complex equilibria from pH titration data has been studied, to establish the conditions under which the method gives accurate values for free ligand and free metal concentrations in systems containing a variety of complexes, including protonated, hydroxy, mixed ligand and polynuclear complexes. Simulated titration data has been used so that the true values of free ligand and free metal concentrations would be known. Calculation procedures are described for each step in the data evaluation, including procedures for extracting information about the stoichiometry of the complexes from the unique information provided by this method. The effect of various systematic and random errors is also considered.

An automated gravimetric titration system was developed for the purpose of collecting highly accurate potentiometric data suitable for use with the Sarkar-Kruck method. In order that none of the theoretical requirements of the method be violated, the system was also designed to maintain total ligand and metal concentrations constant employing computer controlled addition of concentrated reagents from an auxiliary buret.

The Sarkar-Kruck method and the gravimetric titration apparatus were tested in a study of the complexation of zinc by aspartic acid. A system having simple complexation chemistry was chosen in order to avoid problems in the

assignment of a model to the metal ligand reactions. Many of the calculation procedures developed on the basis of simulated titration data were tested and found to be valid with experimental data. Values for the acid dissociation constants of aspartic acid and the formation constants of complexes between zinc and aspartic acid agree well with those reported in the literature.

The complexation of zinc, cadmium and lead by glutathione was studied using the method and apparatus described above. Contrary to some reports in the literature it was found that at low pH the metals bind to glutathione only through the sulfhydryl group with minor complexation occurring through the glycine carboxyl group. The glutamyl amino group remains protonated. In addition, there was evidence for polymerization reactions occurring through the glycine carboxyl at low pH, and through the glutamyl terminal groups at somewhat higher pH. These reactions have been suggested by spectroscopic data but other workers using potentiometry have avoided consideration of these complicating processes.

ACKNOWLEDGEMENTS

I would like to express my thanks to my supervisor, Dr. D.L. Rabenstein, for his guidance throughout the course of this research.

I also want to thank Dr. B. Kratochvil for his help in developing the automatic weight buret, and for reading and commenting on the rough draft of this thesis.

I am also grateful to my wife, Maria, for her patience, encouragement and assistance during the preparation of this thesis.

Financial support from the National Research Council of Canada and the University of Alberta is gratefully acknowledged.

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CHAPTER I

INTRODUCTION

The chemistry of complexation reactions has been the subject of considerable research in the past because of their importance in analytical methods (1), and more recently because of interest in the role which metal ions play in biochemical systems. The methods which have been used to determine formation constants include a variety of spectroscopic methods, calorimetry, reaction kinetics, liquid-liquid partition, solubility measurements, ion exchange, potentiometry, polarography, conductivity, light scattering, osmosis, electrophoresis and many more (2,3,4). Of these, potentiometric titrations with a pH electrode have probably been the most frequently used, and are generally considered to give good results. The reasons for this popularity include: (1) the applicability of the pH titration method to a wide variety of complexes, (2) the relative simplicity of the experiment and the reliability of the data, and (3) the straightforward relationship between the experimentally measured quantities and the chemistry in the solution. A carefully designed and executed experiment will provide a large amount of data, collected under controlled conditions: the concentrations of metals and ligands in solution and the quantity of titrant are accurately known, and the ionic strength and temperature are held constant. The major limitation of the method is that it does not provide any information about the complexes at the molecular level. This means that a model of complexation must be derived intuitively, or by trial and error, or must rely on spectroscopic or other data which gives more specific information at the molecular level.

The focus of this thesis research has been to determine the formation constants for the zinc, cadmium and lead complexes of the tripeptide, glutathione. Glutathione is an important component in the red blood cell and the formation constants of its complexes are of interest for the understanding of the distribution and transport of toxic metals in the body. These complexes have been studied by several investigators, and formation constants reported (5-8). However, there has been no agreement about the model of complexation.

A pH titration method recently proposed by Sarkar and Kruck (9) has the potential for providing information not directly available by any other pH titration method, namely, it derives the concentrations of the free ligand and the free metal from the experimental data without any preconceived model for complexation. The work reported in the first part of this thesis deals with a study and further development of this method, particularly with respect to the design of an experiment which will give the most reliable data. The results of this study indicate that large volumes of highly accurate data are needed for the best application of the method. For this reason an automatic titration system based on a Sartorius electronic balance and a PDP-11/10 minicomputer was designed and built. With this apparatus and the data handling routines developed for the Sarkar-Kruck method, the complexation chemistry of glutathione was studied.

A. The Evaluation of Complex Formation Constants from Potentiometric Titration Data.

The pH titration is a convenient technique with which to collect data reflecting the competition between protons and metal ions for complexation sites on the ligand throughout a wide range of conditions (2,10). Titrations are performed with sample solutions containing several combinations of total metal and total ligand concentrations, and through a wide range of pH. The experimental data is related to the formation constants of the complexes by the mass balance expressions

$$C_H = [H^+] - [OH^-] + \sum p\beta_{pqr}[H]^p[L]^q[M]^r \qquad (1)$$

$$C_L = [L] + \sum q\beta_{pqr}[H]^p[L]^q[M]^r \qquad (2)$$

$$C_M = [M] + \sum r\beta_{pqr}[H]^p[L]^q[M]^r \qquad (3)$$

where C_H is the total concentration of titratable proton and is calculated from the amounts of acids and bases added to the solution, and C_L, C_M are the total ligand and metal concentrations. The formation constant for the complex $H_pL_qM_r$ is β_{pqr} where

$$\beta_{pqr} = \frac{[H_pL_qM_r]}{[H]^p[L]^q[M]^r} \tag{4}$$

The values of C_H, C_L, C_M and the hydrogen ion concentration (from the pH) are known at each data point while the concentrations of ligand and metal, [L] and [M], and the formation constants are unknown. The evaluation of the constants from a set of experimental data may be very simple if only one or two complexes form, but at the other extreme may be very complex if protonated and polynuclear species exist in solution.

A variety of approaches for the handling of the simpler situations have been reported (11,12). These generally are only applicable to very select situations, and very often require additional measurements, for example with an ion selective electrode. The most widely known and applied is an approach described by Bjerrum (13). The $\bar{n}$ method is based upon the calculation of the average number of ligands complexed per metal directly from the experimental data. The method fails, however, if protonated, hydroxy or polynuclear complexes form.

Methods which handle more complex situations fall into two categories, those based on linear statistics and those based on non-linear statistics (2,14). If the data can be arranged into a set of equations such as

$$y = \sum^{N} x_n Q_n \tag{5}$$

where x_n are measured quantities, and Q_n are a set of N unknowns, the constants may be found by a linear least squares calculation to minimize the residual U.

$$U = \sum^{I} (y_i - \sum x_{n_i} Q_n)^2 \tag{6}$$

This method is not generally applicable, and usually requires several extra measurements, aside from pH, to provide suitable values of x_i.

Non-linear methods are very complex, require considerable computer time, and in addition, are reportedly not very reliable (14). The technique, in general, involves systematic adjustment of the formation constants until they provide the best fit to the data. A sequence of calculations will involve:

(a) estimation of the formation constants

(b) with these constants, solve equations (2) and (3) for the unknowns, [L] and [M]. (It must be noted at this point that the values of [L] and [M] are dependent on the model for the complexes and the estimated constants for those species, and may be quite different than the [L] and [M] actually in solution.)

(c) calculate the expected value of an experimentally measured quantity, for example C_H in GAUSS (15,16),

(d) adjust the values of the constants in the direction, and with the proper magnitude to reduce the residual U (Eqn. 7). Return to step (b)

$$U = \sum^{I} (C_{H_i}^{exp} - C_{H_i}^{calc})^2 \qquad (7)$$

until the residual U is minimized.

Steps (b) and (d) may be executed in several ways, and these differences have led to the major data processing computer programs. For example, two approaches have been used to adjust the constants, step (d). The first is based on the first derivative

$$\left(\frac{\partial y}{\partial Q_1}\right) dQ_1 + \left(\frac{\partial y}{\partial Q_2}\right) dQ_2 + \ldots + \left(\frac{\partial y}{\partial Q_N}\right) dQ_N = \text{residual} \qquad (8)$$

and is used in GAUSS (15), SCOGS (17), and MINIQUAD (18). The second is the so-called pit-mapping approach of programs LETAGROP (19) and LETAGROP VRID (20) and is based on a relation similar to Eqn. 8, but in terms of the second derivative. In addition, programs have been written which use both approaches, LEAST (21), and others using a completely different method, STEW (22).

Recently a pH titration method was reported by Sarkar and Kruck (9) which is capable of providing the concentrations of free ligand and free metal throughout the pH range, using only the experimental data from an appropriately designed set of titrations. This is in clear contrast to the methods described above, where [L] and [M] must be calculated using a hypothetical model, and guesses for the formation constants. With the values of [L] and [M] derived experimentally the non-linear problem of solving for formation constants reduces to a linear one, even for systems which include hydroxy and polynuclear complexes. The formation constants may be extracted from Equations 1, 2 or 3 using a linear least squares calculation. Application of this method to a number of metal-amino acid systems has been reported (23-26). Nonetheless it appears that the method has not been proven applicable to polynuclear complexes, and that the exact consequences of error originating in the experimental data, and in the data handling steps have not been described. In addition, the reports published by Sarkar and Kruck did not describe the experimental method in detail. From these reports it appears that no attempt was made to determine the conditions which would give the best experimental results, and as a result it seems that the experimental methods used in these reports may have violated the theoretical requirements of the method.

The main thrust of the work reported here was to simplify the calculations as much as possible, to prove that the method is applicable to the more exotic complexes, and finally to establish the experimental conditions which will give the most reliable data possible. The results of this study are described in Chapter II.

B. Automatic Titration Apparatus.

Of the many automatic solution handling methods available, the most popular are those based on a stepping motor driven piston buret. Several units are available commercially, including the Mettler DV11 and the Radiometer ABU13, and many workers have devised their own (27-34), for example using a commercially available micrometer driven buret such as the Gilmont ultra precision buret, powered by a stepping motor. These types of systems are very easy to automate; a delivery of a known volume only requires a series of electrical pulses. They are however, high in price, and usually low in precision. This lack of accuracy usually stems from imperfection in the mechanical drive from the stepping motor to the piston, and the usual problems with volumetric methods, including temperature effects, out of calibration glassware, leaks, and so on.

Only a few alternatives to the piston buret have been reported. Coulometric generation of titrant is an old, and potentially very precise method(35). Its general application to high precision acid-base titrations has been limited by two factors; first, the titrant generation reaction must be very efficient and allow high current densities and, second, the working electrode must be isolated from the sample solution if the sample contains electroactive species (36). Coulometric methods can be easily automated (37).

A unique form of real time titrator has been reported (38), in which the sample and an added colour indicator is passed through a narrow dialysis tube within a bath of titrant. The titrant diffuses into the tube and the point where the sample is neutralized is marked by the colour change of the indicator.

A titrator based on the delivery of a series of sub-microlitre droplets (39,40) was reported by Hieftje (41,42). In this system, titrant was forced through a capillary which was vibrated with a piezoelectric crystal. This dispersed the liquid into individual droplets, which the authors ingeniously counted, and directed into a sample solution using electrostatic forces. Another variation was reported (43) in which the time during which the droplets were allowed into the sample was measured instead of the number. Both of these systems suffered from very poor precision.

The handling of solutions on the basis of weight has long been regarded as superior to any type of volumetric technique. However, due to the slowness of the weighing operation, and the virtual impossibility of automation, gravimetric procedures have not gained much popularity. The advantages of gravimetric titrations over volumetric methods include:

(a) elimination of drainage, reading, and temperature change error inherent to volumetric equipment,

(b) elimination of time consuming gravimetric calibration of volumetric equipment,

(c) ability to measure and dispense accurately known weights of volatile or highly viscous liquids,

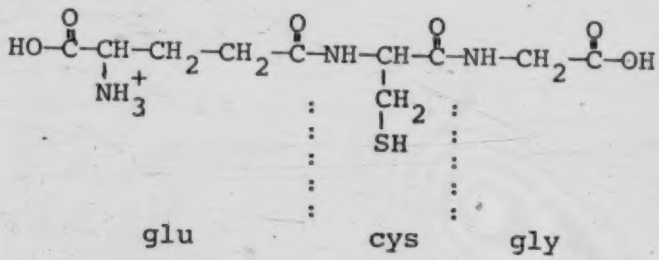
(d) ability to measure with high precision small quantities of solution for microchemical work, or with costly or scarce materials.

Manual methods have been described for special situations (44,45), including work with non-aqueous solvents, and where particularly good precision was desired (46). Malmstadt (47) has reported an automatic system in which the weight of reagent delivered is determined by weighing the container into which the material is dispensed. This method is not suitable for a titration in the usual manner because no provision is made, or seems possible, for monitoring a sample property, for example the pH.

In this thesis a completely automated gravimetric titration system is described, based on a Sartorius electronic balance and a PDP-11/10 mini computer. The delivery of reagent from a reservoir located on the balance pan was initiated by an optical device. After delivery, the weight of titrant delivered was known by the decrease in weight of the unit on the balance pan. This system combined the accuracy and precision of a technique based on a weighing procedure, with the speed and reliability of automation. Gravimetric titrations were run with high precision, and in a fraction of the time needed for equivalent manual operations. This system will be described in considerable detail in Chapter IV.

C. The Complexation Chemistry of Glutathione.

Glutathione is a tripeptide composed of the amino acids glutamic acid, cysteine, and glycine.



$$\mathrm{HO{-}\overset{O}{\overset{\|}{C}}{-}\underset{NH_3^+}{\underset{|}{CH}}{-}CH_2{-}CH_2{-}\overset{O}{\overset{\|}{C}}{-}NH{-}\underset{\underset{\displaystyle SH}{|}}{\underset{CH_2}{\underset{|}{CH}}}{-}\overset{O}{\overset{\|}{C}}{-}NH{-}CH_2{-}\overset{O}{\overset{\|}{C}}{-}OH}$$

The binding to metal ions may occur through the carboxyl and amino groups of the glutamyl terminal, through the sulfhydryl group, and through the glycine carboxyl group. In addition, it has been suggested that some binding may occur through protonated and deprotonated peptide linkages (5,6). With this number of binding sites, and the number of groups which are protonated at various pH ranges, the complexation chemistry of glutathione is not simple.

The earliest attempts to unravel the complexation of glutathione (8,48-50) relied upon relatively simple pH titration experiments, and on polarography, but the data handling methods were not sufficiently advanced to handle protonated and polynuclear complexes. The development of spectroscopic techniques such as NMR allowed the study of metal-ligand interaction at the molecular level, for example indicating which groups were bound to the metal.

The sulfhydryl group is known to be the most active in the complexation of glutathione to a number of metals, binding very strongly to "soft" metals such as mercury, lead and cadmium and less strongly to zinc, and other transition elements (7,50,51,52). The interaction of mercury with the sulfhydryl is particularly strong, and the metal ion can only be displaced by protons at very low pH. Since the carboxyl and amino groups do not bind as strongly, several protonated complexes exist at low pH (51). Other mercury complexes, containing as many as three metal ions and two glutathione molecules have been shown to exist (50, 51).

Studies of the binding of glutathione to other metals, including Cu, Ni, Fe and Pd (53-55) have indicated that the sulfur is again the primary binding site. Complexation does occur through the glutamyl and glycine residues as well, but it has not been possible to determine if those groups are bound to the same metal ion as the sulfhydryl, or to another. It has been pointed out that the complexation of the glutamyl and sulfhydryl groups to the same metal would form an unstable ten membered ring, indicating that at least in some cases, a polymerization process is likely to occur (7).

The development of non-linear methods of treating pH titration data made possible a more detailed analysis of the composition of a solution containing a mixture of metal ions and glutathione (5,6). The derivation of a model of complexation has, however, been largely a matter of intuition and guesswork, and the results of studies involving zinc, cadmium and lead do not agree with each other nor do they agree with the results of NMR experiments (7). Since pH titration methods do not, in general, provide any information about the types of complexes existing in solution, the choice of a model must be guided by some other evidence. The Sarkar-Kruck method provides, unlike other methods, experimental values for [L] and [M] which, as is discussed in Chapter II, allows some rational deduction of a model of complexation. In addition, the number of models that will fit the data is considerably reduced. For these reasons it was hoped that the Sarkar-Kruck method would produce more meaningful results about the complexation of glutathione than have been thus far reported. The results of the metal-glutathione experiments and the discussion of models for complexation appear in Chapter VI.

CHAPTER II

A STUDY OF THE OSTERBERG-SARKAR-KRUCK METHOD OF EVALUATING FREE METAL AND FREE LIGAND CONCENTRATIONS IN SOLUTIONS OF COMPLEX EQUILIBRIA

A. Introduction.

Sarkar and Kruck recently described a pH titration method in which the free metal ion and the free ligand can be calculated simultaneously throughout the central titration of an appropriately designed set of titrations. This method is appealing in that the concentrations are obtained directly from the experimental data, without assuming any hypothesized collection of species for that system. The calculation of stability constants then reduces to a linear least squares problem using any of the mass balance equations. If the stoichiometries of the complexes are not known, it still is necessary to postulate a model. It is to be expected, however, that fewer models will fit the data because the free ligand and the free metal concentrations are known and are not derived from the model.

In this chapter a critical examination of this approach is described. The emphasis of this examination has been to establish the conditions under which the method gives accurate values for the free metal and the free ligand concentrations in systems containing a variety of complexes, including protonated, hydroxy, polynuclear and mixed ligand complexes. The approach has been to analyze simulated titration data, so that the true values of free metal and free ligand concentration would be known. In addition, since the method provides information not available in the study of complex systems by other data evaluation procedures, ways in which this information can be used to provide guidance in the selection of a model are considered.

This chapter refers exclusively to simulated titration data based on several hypothetical complexation systems. The complexation systems and the parameters used in the data simulation are summarized in Tables 1-5. Series A and B (Table 1) are for a polyprotic acid, Series C (Table 2) is for a triprotic ligand and the complexes HML, HML_2 and ML, and Series D (Table 3) is for a diprotic ligand and the complexes ML, ML_2, ML_3 and $H_{-1}ML$. Series E (Table 4) includes polynuclear species and Series F (Table 5) includes mixed ligand complexes. In the following discussions, examples of calculation methods and the effects of error will be based on these series of titrations. The computer program used to simulate the titrations of Series A through F is shown in Appendix A.

Overview of the Method

This method provides the free concentrations of ligand

Table 1. Simulation Parameters: Series A and B

Complexes	Formation Constants β
H_4L	1.0×10^{25}
H_3L	1.0×10^{22}
H_2L	1.0×10^{18}
HL	1.0×10^{10}

Titration	Total Ligand C_L	Initial pH	pH Interval	Titrant Concentration, M
A_1	8.0×10^{-4}	3.00	0.05	1.00
A_2	9.0×10^{-4}	3.00	0.05	1.00
A_3	1.0×10^{-3}	3.00	0.05	1.00
A_4	1.1×10^{-3}	3.00	0.05	1.00
A_5	1.2×10^{-3}	3.00	0.05	1.00
B_1	8.0×10^{-4}	3.414	0.075	0.1800
B_2	9.0×10^{-4}	3.406	0.066	0.2011
B_3	1.0×10^{-3}	3.399	0.071	0.220
B_4	1.1×10^{-3}	3.411	0.056	0.1567
B_5	1.2×10^{-3}	3.410	0.068	0.2500

Table 2. Simulation Parameters: Series C

Complexes	Formation Constants
H_3L	1.0×10^{21}
H_2L	1.0×10^{16}
HL	1.0×10^{10}
HML	1.0×10^{17}
HML_2	1.0×10^{25}
ML_2	1.0×10^{18}

Series C: No Dilution

pH Range: 3.0 - 10.0

pH Interval: 0.1 (71 data points)

Initial Volume: 300 ml

Titrant Concentration: no titrant

Middle Titration: $C_L = 3.0 \times 10^{-3}$, $C_M = 1.0 \times 10^{-3}$

Titration	C_L	C_M
C_1	2.99×10^{-3}	1.00×10^{-3}
C_2	3.00×10^{-3}	1.00×10^{-3}
C_3	3.01×10^{-3}	1.00×10^{-3}
C_4	3.00×10^{-3}	9.9×10^{-4}
C_5	3.00×10^{-3}	1.01×10^{-3}

C' Series: With Dilution

As above except

Titrant Concentration: 0.4 M NaOH

Table 3. Simulation Parameters: Series D

Complexes	Formation Constants
H_2L	1.0×10^{10}
HL	1.0×10^{6}
ML	1.0×10^{6}
ML_2	1.0×10^{11}
ML_3	1.0×10^{15}
$H_{-1}ML_2$	1.0×10^{4}

pH Range: 3.0 - 10.0

pH Interval: 0.1 (71 data points)

Initial Volume: 300 ml

Titrant Concentration: no titrant

Middle Titration: $C_L = 4.0 \times 10^{-3}$, $C_M = 1.0 \times 10^{-3}$

Titration	C_L	C_M
D_1	3.6×10^{-3}	1.0×10^{-3}
D_2	3.9×10^{-3}	1.0×10^{-3}
D_3	4.0×10^{-3}	1.0×10^{-3}
D_4	4.1×10^{-3}	1.0×10^{-3}
D_5	4.4×10^{-3}	1.0×10^{-3}
D_6	4.0×10^{-3}	9.0×10^{-4}
D_7	4.0×10^{-3}	9.5×10^{-4}
D_8	4.0×10^{-3}	1.05×10^{-3}
D_9	4.0×10^{-3}	1.10×10^{-3}

Table 4 . Simulation Parameters: Series E

Complexes	Formation Constants
H_3L	1.0×10^{21}
H_2L	1.0×10^{16}
HL	1.0×10^{10}
H_2ML	1.0×10^{19}
HM_2L	1.0×10^{18}
M_2L_2	1.0×10^{17}

pH Range: 3.0 - 10.0

pH Interval: 0.1 (71 data points)

Initial Volume: 300 ml

Titrant Concentration: no titrant

Middle Titration: $C_L = 2.5 \times 10^{-3}$; $C_M = 1.0 \times 10^{-3}$

Titration	C_L	C_M
E_1	2.0×10^{-3}	1.0×10^{-3}
E_2	2.2×10^{-3}	1.0×10^{-3}
E_3	2.4×10^{-3}	1.0×10^{-3}
E_4	2.5×10^{-3}	1.0×10^{-3}
E_5	2.6×10^{-3}	1.0×10^{-3}
E_6	2.8×10^{-3}	1.0×10^{-3}
E_7	3.0×10^{-3}	1.0×10^{-3}
E_8	2.5×10^{-3}	8.0×10^{-4}
E_9	2.5×10^{-3}	9.0×10^{-4}
E_{10}	2.5×10^{-3}	9.5×10^{-4}
E_{11}	2.5×10^{-3}	1.05×10^{-3}
E_{12}	2.5×10^{-3}	1.10×10^{-3}
E_{13}	2.5×10^{-3}	1.20×10^{-3}

Table 5 . Simulation Parameters: Series F

Complexes	Formation Constants
H_3L	1.0×10^{20}
H_2L	1.0×10^{16}
HL	1.0×10^{10}
ML	1.0×10^{9}
H_2L'	1.0×10^{15}
HL'	1.0×10^{8}
ML'	5.0×10^{7}
HMLL'	1.0×10^{22}
MLL'	1.0×10^{14}

pH Range: 3.0 - 10.0

pH Interval: 0.1 (71 data points)

Initial Volume: 300 ml

Titrant Concentration: no titrant

Middle Titration: $C_L = 2.0 \times 10^{-3}$, $C_{L'} = 2.0 \times 10^{-3}$, $C_M = 1.0 \times 10^{-3}$

Titration	C_L	$C_{L'}$	C_M
F_1	1.4×10^{-3}	2.0×10^{-3}	1.0×10^{-3}
F_2	1.6×10^{-3}		
F_3	1.8×10^{-3}		
F_4	2.0×10^{-3}		
F_5	2.2×10^{-3}		
F_6	2.4×10^{-3}		
F_7	2.6×10^{-3}		

Table 5 (continued)

Titration	C_L	$C_{L'}$	C_M
F_8	2.0×10^{-3}	1.4×10^{-3}	1.0×10^{-3}
F_9		1.6×10^{-3}	
F_{10}		1.8×10^{-3}	
F_{11}		2.2×10^{-3}	
F_{12}		2.4×10^{-3}	
F_{13}		2.6×10^{-3}	
F_{14}	2.0×10^{-3} ,	2.0×10^{-3}	0.7×10^{-3}
F_{15}			0.8×10^{-3}
F_{16}			0.9×10^{-3}
F_{17}			1.1×10^{-3}
F_{18}			1.2×10^{-3}
F_{19}			1.3×10^{-3}

and metal in solutions of complex equilibria, and for simplicity in this discussion will be called FICS, for Free Ion Concentrations in Solution. The basic relationships in this method are

$$p[L] = p[L]_o - \int_{pH_o}^{pH} \left(\frac{dC_H}{dC_L}\right)_{C_M, pH} dpH \qquad (9)$$

$$p[M] = p[M]_o - \int_{pH_o}^{pH} \left(\frac{dC_H}{dC_M}\right)_{C_L, pH} dpH \qquad (10)$$

where C_H is the concentration of titratable proton, C_L and C_M are the total analytical concentrations of ligand and metal respectively, and [L] and [M] are their free concentrations. Alternate derivations of Equations 9 and 10 may be found in references 9 and 56. An abbreviated example may be found in Appendix B.

For systems containing more components, equations analogous to 9 and 10 can be written for each component in the solution. For simplicity let us only consider the component L, remembering that the discussion is applicable to every component in the system.

The quantity $p[L]_o$ is the negative logarithm of the concentration of free L at some initial pH_o. A pH_o is usually chosen at which no complex is formed, and consequently $[L]_o$ is equal to (or may be calculated from) C_L. The integral of $(dC_H/dC_L)_{C_M,pH}$ is evaluated from pH_o to the pH at which p[L] is to be evaluated.

One experimental method for obtaining $(dC_H/dC_L)_{C_M,pH}$ as a function of pH is with a series of titrations where the total concentration of L is varied, while all the other components are at constant concentration. At a given pH, C_H is extracted from each of the titrations. The derivative $(dC_H/dC_L)_{C_M,pH}$ is the change in C_H with C_L, measured at C_L of the middle titration. In exactly this way the derivative at each pH throughout the titration is calculated. The values of $(dC_H/dC_L)_{C_M,pH}$ are integrated from pH_o to a given pH, and p[L] at that pH is then calculated from Equation 9. The experiment is designed in such a way that, given a system of composition C_L and C_M, referred to as the composition of the middle titration, each component is in turn selectively varied. The FICS method will then provide [L] and [M] as a function of pH, for a solution of that overall composition.

The stability constants of the complexes are calculated by a linear least squares fit of the [L] and [M] data into the mass balance equations. Any one mass balance is completely sufficient unless a given complex does not contain any of that particular component.

In the following section, the calculation procedures at each step in the method are considered.

B. Calculation Procedures in the FICS Method.

C_H Calculations

The extraction of useful information from the pH titration requires accurate knowledge of several parameters. The FICS method requires the total ligand concentration, C_L, the total metal concentration, C_M, the total concentration of mineral acid added, C_A, the pH and the concentration of added base, C_B. All of these quantities change during a conventional titration and careful work is needed to provide accurate knowledge of their values.

The first quantity extracted from the raw data is the total concentration of titratable proton, C_H.

$$C_H = (N_{DP} \times C_L) + C_A - C_B \qquad (11)$$

where N_{DP} is the number of disposable protons on the ligand. In order that the FICS method be used, C_H must be known at closely spaced pH values throughout the titrations, and moreover, it must be known at the same pH's in each of a series of titrations. Since this is difficult experimentally, some manipulation of the original data is necessary to find these C_H values. Several possibilities exist, including the fitting of the pH-C_H data to a mathematical function, perhaps a type of polynomial, and in subsequent steps extracting C_H at any needed pH. This is the approach taken by Sarkar and Kruck (9). However, in our hands a program of this type obtained from other workers did not function very well. It was found that linear interpolation between existing data points was sufficient to provide C_H at specific pH's throughout the titration. Efficient use of experimental data means fixing a pH interval approximately equal to that of the available data.

The linear interpolation is limited in two ways; first it does not use the entire body of data as a unit, and therefore does not have the smoothing potential which other procedures may have, and second it will bias the data in some cases. A bias to large C_H will result if the C_H vs pH curve is concave upward, and to low C_H's if the curve is concave downward. In most cases this will give very small errors at pH intervals of about 0.1 units.

The effect of linear interpolation on the final results of FICS is illustrated in Table 6. Series A uses C_H's directly from the simulation program, and Series B uses C_H's calculated by interpolation on data generated at a varying set of pH's. Possible error was encouraged in the second set by increasing the pH intervals from 0.05 to 0.1 and by increasing the dilution by titrant (lowered NaOH concentration). The simulation parameters for Series A and B appear in Table 1.

Table 6. The Results of the FICS Calculations on the Simulated Titration Data, Series A and B

	Free Ligand		
pH	Known	A Series[a]	B Series[a] (interpolation)
3.5	1.937×10^{-15}	1.937	1.937
4.0	4.761×10^{-14}	4.762	4.761
4.5	7.537×10^{-13}	7.539	7.543
5.0	9.075×10^{-12}	9.077	9.084
5.5	9.661×10^{-11}	9.666	9.675
6.0	9.802×10^{-10}	9.807	9.817
6.5	9.662×10^{-9}	9.666	9.676
7.0	9.080×10^{-8}	9.084	9.091
7.5	7.588×10^{-7}	7.591	7.593
8.0	4.974×10^{-6}	4.975	4.975
8.5	2.346×10^{-5}	2.347	2.347
9.0	8.331×10^{-5}	8.335	8.338

		Calculated β	
Complex	Known β	A Series	B Series
H_4L	1.0×10^{25}	9.9974×10^{24}	9.9790×10^{24}
H_3L	1.0×10^{22}	1.0003×10^{22}	1.0013×10^{22}
H_2L	1.0×10^{18}	9.9973×10^{17}	9.9885×10^{17}
HL	1.0×10^{10}	1.0004×10^{10}	9.9992×10^{9}

[a] Calculated [L] and expected [L] are identical in order of magnitude.

The Derivative $(dC_H/dC_X)_{C_Z,pH}$

The second calculation in the FICS procedure is the evaluation of the derivative $(dC_H/dC_X)_{C_Z,pH}$. The quantity C_H has been discussed. C_X will be used to denote the total analytical concentration which is varied. This may be C_L, the ligand concentration, or C_M, etc. The term C_Z represents all the other total concentrations which are fixed.

The experimental determination of this derivative is the most challenging step of the FICS procedure. The straightforward experiment is to perform several titrations varying one C_X, perhaps C_L, while maintaining C_M and any other concentrations constant. The simulation parameters for Series D, shown in Table 3 illustrates this more clearly. At some pH, for example 4.0, C_H is calculated from each of the titrations. This gives the variation in C_H with C_L at pH 4.0, or $(dC_H/dC_L)_{C_M,pH\ 4.0}$.

The accuracy of this quantity depends on several factors:

(1) the accuracy of C_H and C_L,

(2) the curvature of the C_H vs pH function,

(3) the number of titrations, and therefore the number of C_H's available,

(4) the degree to which C_Z (the other components in solution) remains constant.

The function C_H vs C_X, and the effects of problems (1) to (4) must be clearly understood in order to obtain the best results from the FICS method.

The variation of C_H with C_L for Series D, which consists of a diprotic ligand and the complexes ML, ML_2, ML_3 and $H_{-1}ML_2$ is shown in Figure 1. The total metal concentration was fixed at 1.0 x 10^{-3}, and curves drawn at several pH values. The simulation parameters for Series D may be found in Table 3.

Several characteristics of the C_H vs C_L function can be seen in Figure 1, including severe curvature in some regions, and reasonable linearity at excesses of C_L (C_M was 1.0 x 10^{-3}). In addition it should also be noted that C_H may vary over a wide range of concentrations, and tends to be small at low ligand concentrations. Several practical considerations can be based on these observations. Titration conditions must be chosen to avoid areas of curvature. Each point on the C_H vs C_L plot represents a data point from a separate titration, and the number of titrations may be limited. A reasonable estimate of $\Delta C_H/\Delta C_L$ requires therefore that C_H vary linearly with C_L, and , in addition, there is some advantage to having ΔC_H of a reasonable magnitude. If the change in C_H with C_L is linear, only two titrations are really necessary, and more will improve the situation. Reasonable random error is acceptable since a linear least squares fit will tend to give better derivatives than the limit of accuracy of C_H might

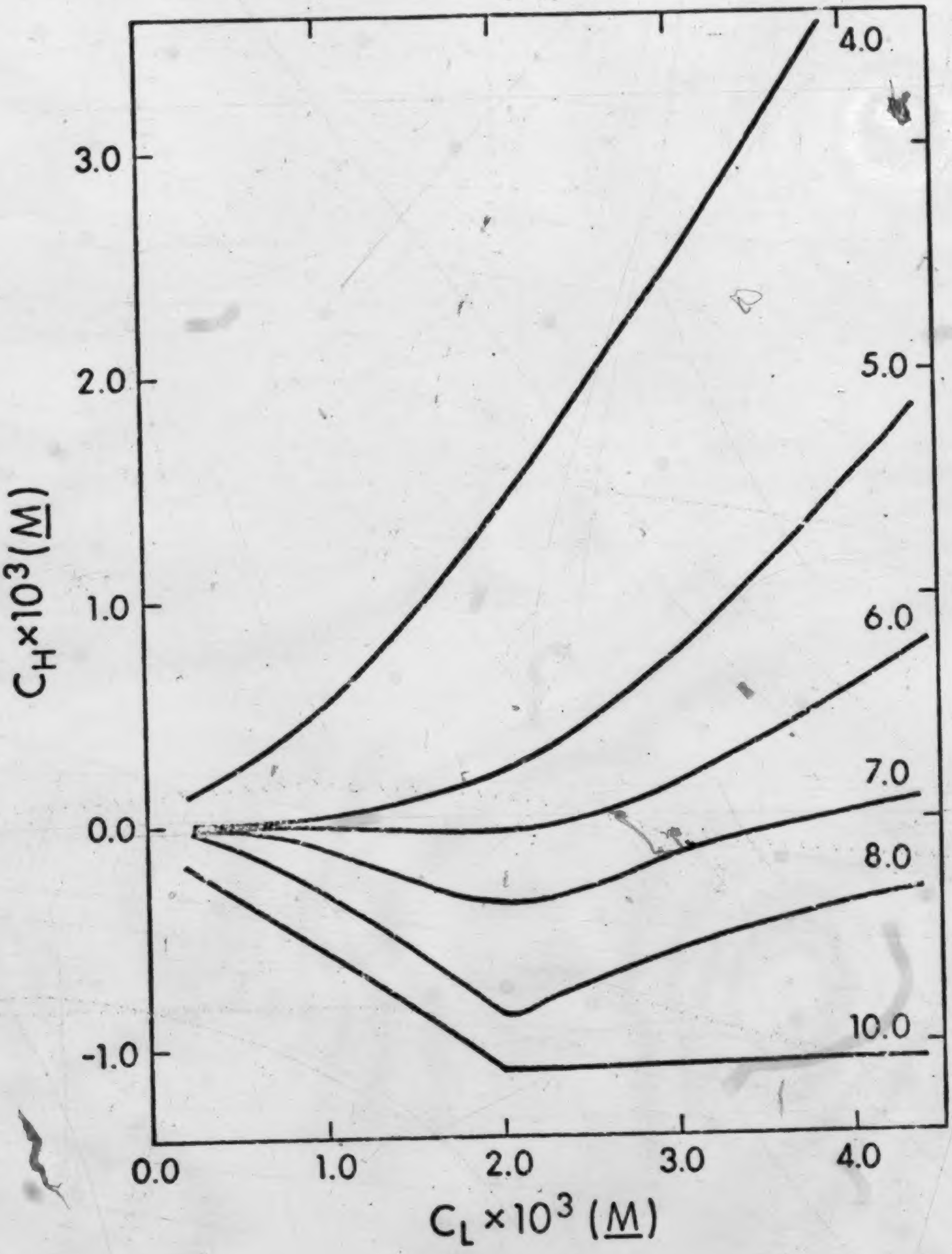


Figure 1. C_H vs C_L for a system containing a diprotic ligand and complexes ML, ML_2, ML_3 and $H_{-1}ML_2$. The overall ligand protonation constants are 1.0×10^6 and 1.0×10^{10}. The formation constants of the complexes are 1.0×10^6, 1.0×10^{11}, 1.0×10^{15} and 1.0×10^4 respectively. C_M was constant at 1.0×10^{-3} M. (See Table 3.)

indicate possible.

A least squares polynomial fit of C_H vs C_L data will not improve the situation. First, if the function of C_H vs C_L is linear and has some random error, the polynomial will try to fit even those errors, and second, if the function of C_H vs C_L is nonlinear it is not likely to be as simple as a polynomial of low degree and a systematic distortion of the actual relation will occur.

Consideration must also be given to problems which may arise in titrations where the C_H may become small. This will happen if the ligand concentration is low, or if the metal concentration is high. Since C_H is calculated from the difference in two large numbers, the total acid added to the solution and the total base added, it may lose significance in some parts of the titration curves. No problem is encountered if only one out of several C_H values at different C_L's is small, which is a common situation, but difficulty is encountered if the C_H's corresponding to several C_L values are small simultaneously. Each C_H has limited significance, and therefore the ΔC_H in the $(\Delta C_H/\Delta C_L)$ quantity has low precision. In general, small values of (dC_H/dC_L) and (dC_H/dC_M) will always have low precision, but since they also contribute less information about complexation in solution they should be avoided.

In summary, with data of limited accuracy and in limited numbers, the best results are obtained if the C_H's are of reasonable magnitude, if (dC_H/dC_X) is of reasonable magnitude and if the change in C_H with C_X is linear.

Figure 1, mentioned above, is the variation in C_H with C_L for Series D, including the complexes ML, ML_2, ML_3 and $H_{-1}ML_2$. At pH 4.0 very little complexation is occurring and C_H increases continuously with increasing ligand concentration. Each increase of C_L adds protonated ligand species to the solution, and therefore the concentration of titratable protons, C_H, increases. Above pH 5.0 complexation becomes appreciable. At C_L concentrations below 2.0×10^{-3} M, changes in the ligand concentration have very little effect on C_H. This is because the ligand is bound as ML and ML_2 complexes, which have no titratable protons. Near pH 6.0 this situation is severe, and the problems discussed above may become significant; C_H is nearly zero, and changes only slightly with C_L. This problem is avoided if the titrations are performed at an excess of ligand, in this case about 4.0×10^{-3} M.

The curves above pH 7.0 have two regions. Below a C_L of 2.0×10^{-3} M, the changes in C_H with C_L are governed by the complex $H_{-1}ML_2$. Increasing C_L from 1.0×10^{-3} to 2.0×10^{-3} will increase the concentration of $H_{-1}ML_2$, and since this is a hydroxy species, it effectively contributes a negative number of titratable protons, and C_H becomes more negative as the concentration of $H_{-1}ML_2$ increases. Beyond a C_L of 2.0×10^{-3} no further $H_{-1}ML_2$ may form since the metal is completely coordinated. Addition of ligand contributes protonated HL species to the solution, and C_H

increases. At pH 10.0 the ligand is completely deprotonated and C_H no longer increases with C_L beyond 2.0×10^{-3} M.

Figure 2 shows the C_H vs C_L plots for a system similar to Series C, composed of H_3L, H_2L, HL,HML, HML_2 and ML_2. The details of the simulation parameters for these titrations are shown in Table 7. At pH 4.0 only slight complexation is occurring ; increasing C_L adds protonated ligand to the solution, and C_H rises continously. At pH 7.0 however, complexation is occurring and the change in C_L with C_L is more complicated. At C_L below 1.0×10^{-3} M, C_H rises with increasing C_L because each addition of C_L increases the concentration of the complex HML. No other complex may form because there is a large excess of metal in those regions ($C_M = 1.0 \times 10^{-3}$). When C_L becomes larger than 1.0×10^{-3} each addition of ligand converts HML species to HML_2 and ML_2. The only reason HML existed was because there was insufficient ligand to form HML_2 and ML_2. The species ML_2 has no protons and therefore C_H falls with increasing C_L in the range 1.0×10^{-3} to 2.0×10^{-3} M of ligand concentration. Beyond a C_L of 2.0×10^{-3} M, the addition of ligand in excess of that needed to coordinate the metal adds protonated ligand species to the solution, and C_H increases continuously. At pH 9.5 no HML species will form, and the maximum in C_H at a C_L of 1.0×10^{-3} has disappeared. In this case all of the added ligand is immediately converted to ML_2 in spite of the excess metal. Beyond the complete coordination of the metal, the C_H rises

Table 7. Simulation Parameters: Variation of C_H with C_L and C_M for Figures 2 and 3

Complexes	Formation Constants
H_3L	1.0×10^{20}
H_2L	1.0×10^{16}
HL	1.0×10^{10}
HML	1.0×10^{16}
HML_2	1.0×10^{24}
ML_2	1.0×10^{17}

pH Range: 3.0 - 10.0

pH Interval: 0.1 (71 data points)

Initial Volume: 300 ml

Titrant Concentration: no titrant

Component	Figure 2	Figure 3
C_L	varied	4.0×10^{-3} M
C_M	1.0×10^{-3} M	varied

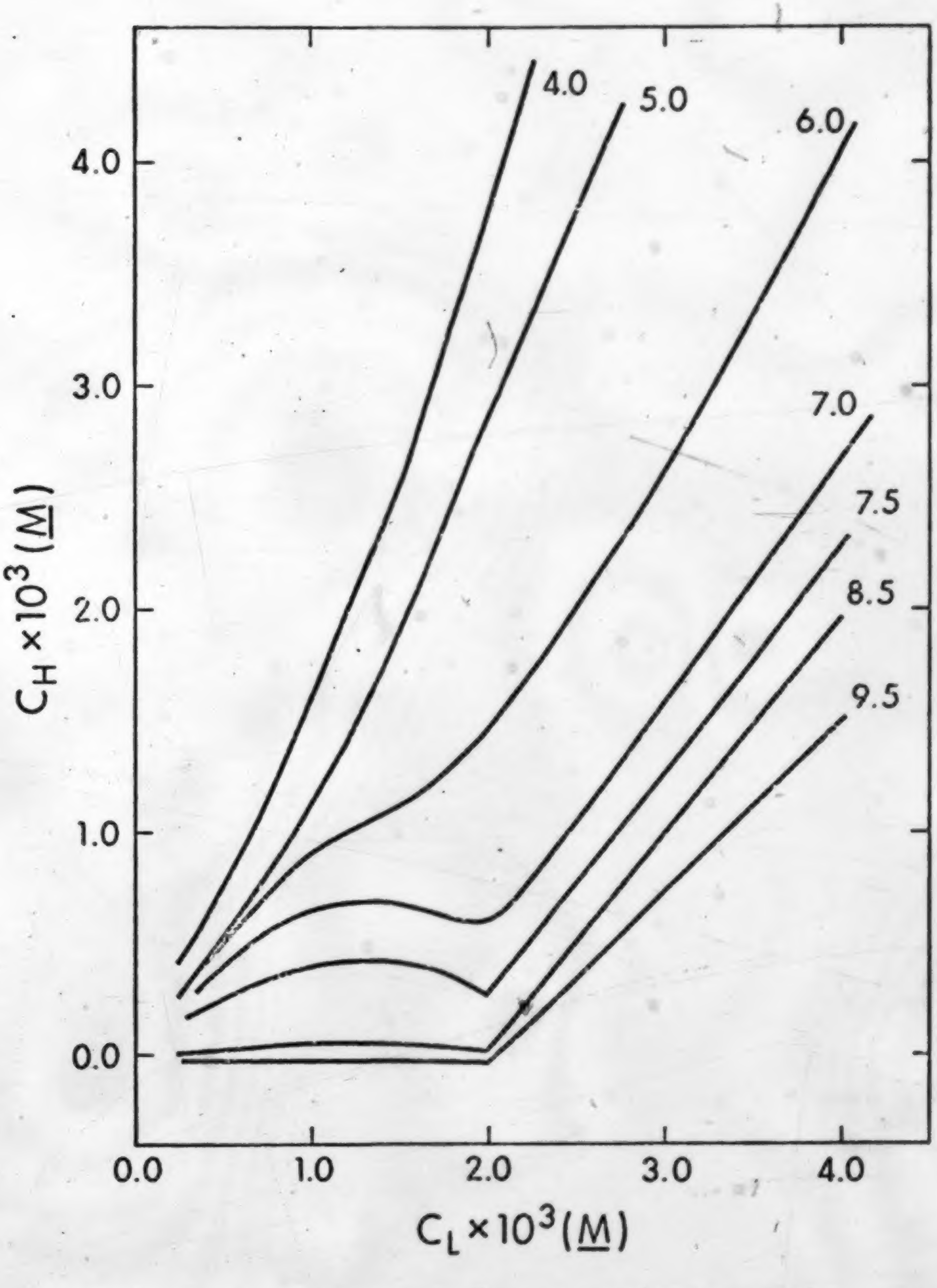


Figure 2. C_H vs C_L for a system containing a triprotic ligand and complexes HML, HML_2, and ML_2. The overall ligand protonation constants are 1.0×10^{10}, 1.0×10^{16} and 1.0×10^{20}. The formation constants of the complexes are 1.0×10^{16}, 1.0×10^{24} and 1.0×10^{17} respectively. C_M was constant at 1.0×10^{-3} M.

due to the addition of protonated ligand to the solution.

The C_H vs C_M plots for the same system as shown in Figure 2 are shown in Figure 3. The total ligand concentration is fixed at 4.0×10^{-3} $\underline{M}$, so that the far right hand side of the figure is a 1:1 ligand to metal ratio. At pH 4.0 the values of C_H decrease continuously as C_M is increased. This occurs because each addition of metal converts some of the ligand, perhaps present as H_3L or H_2L, to the complex HML. The average number of protons available to each ligand therefore decreases and C_H decreases as well. At pH 7.0 the curve has two regions. Below a metal concentration of 2.0×10^{-3} $\underline{M}$ there is a large excess of ligand (C_L was 4.0×10^{-3} $\underline{M}$) and each addition of metal favours the formation of ML_2. Ligands which were in the H_2L and [illegible] form are converted to ML_2, and again the average number of protons available to each ligand decreases. Beyond a metal concentration of 2.0×10^{-3} each addition of metal favours the production of HML, and liberates ligands from the ML_2 complexes, and C_H begins to increase with addition of metal. At pH 9.5, as mentioned above, the only complex which is possible is ML_2. At a low metal concentration each addition of the metal converts free protonated ligand species to ML_2 and C_H falls. Beyond a C_M of 2.0×10^{-3} $\underline{M}$ all of the ligand is held as ML_2 and each addition of metal only adds free uncomplexed metal to the solution, which has no effect on C_H.

These plots indicate that there are regions where the

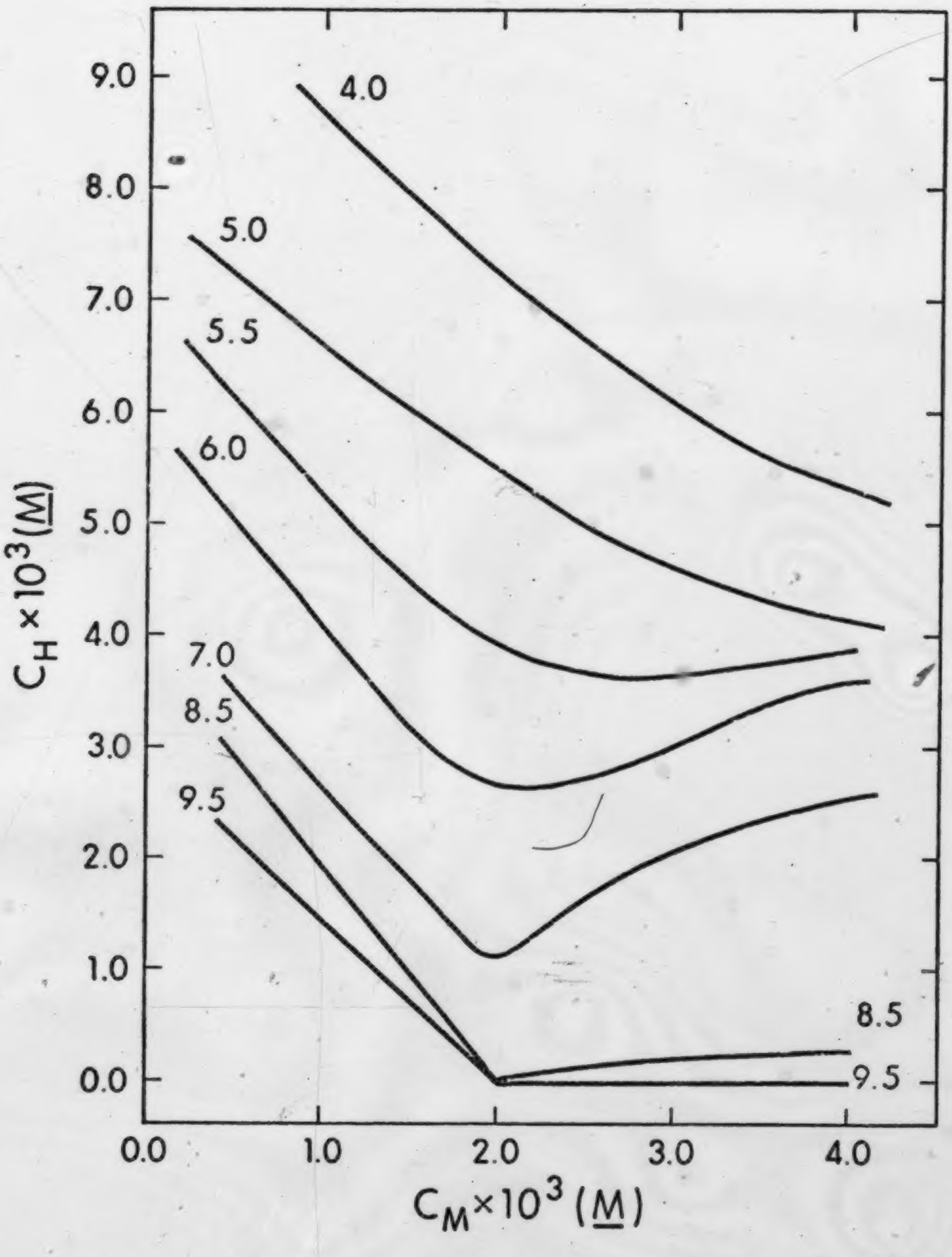


Figure 3. C_H vs C_M for the system of complexes defined in the legend of Figure 2 . C_L was constant at 4.0×10^{-3}M.

C_H vs C_L or C_H vs C_M functions may have severe curvature, and though such areas are of interest to investigate the complexes forming in solution, and potentially have a large amount of information in them, difficulty is encountered in experiments designed to give (dC_H/dC_L) and (dC_H/dC_M). In these cases linear regions are preferred. C_H vs C_L and C_H vs C_M will be linear if the ligand concentration is sufficiently high that the metal coordination is completely satisfied at all pH's. In other words, the C_L to C_M ratio should be larger than the L to M ratio within the complexes predominating in any pH range.

In summary, $(dC_H/dC_X)_{C_Z,pH}$ may be difficult to evaluate with reliability throughout wide pH ranges. The most severe problems are avoided if:

(1) C_H is maintained at a reasonable absolute magnitude
(2) (dC_H/dC_X) are of a reasonable absolute magnitude
(3) regions of curvature in C_H vs C_X are avoided.

The Integral

The values of $(dC_H/dC_X)_{C_Z,pH}$ usually fall within $\pm n$ where n is the number of protons on the fully protonated ligand. There are no regions of extreme curvature as seen on the C_H vs C_X plots. Mathematical treatment of these curves is therefore straightforward. Figure 4 shows the values of (dC_H/dC_L) and (dC_H/dC_M) for Series D (Table 3) at a C_L of 4.0×10^{-3} and a C_M of 1.0×10^{-3} $\underline{M}$. Figure 5

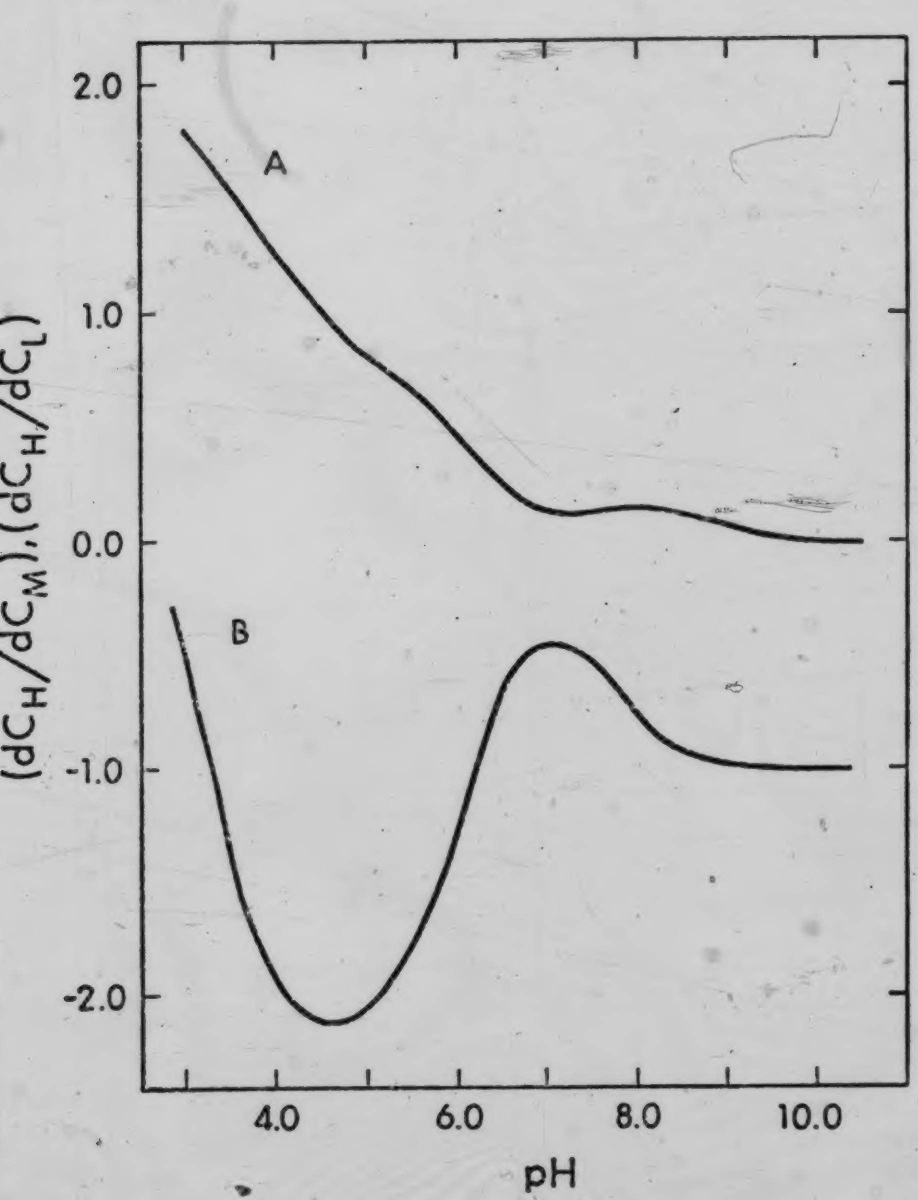


Figure 4 . $(dC_H/dC_L)_{C_M,pH}$, A; and $(dC_H/dC_M)_{C_L,pH}$, B; as a function of pH for the system of complexes defined in the legend of Figure 1 . $C_M = 1.0 \times 10^{-3}$M, $C_L = 4.0 \times 10^{-3}$M. (See Table 3 .)

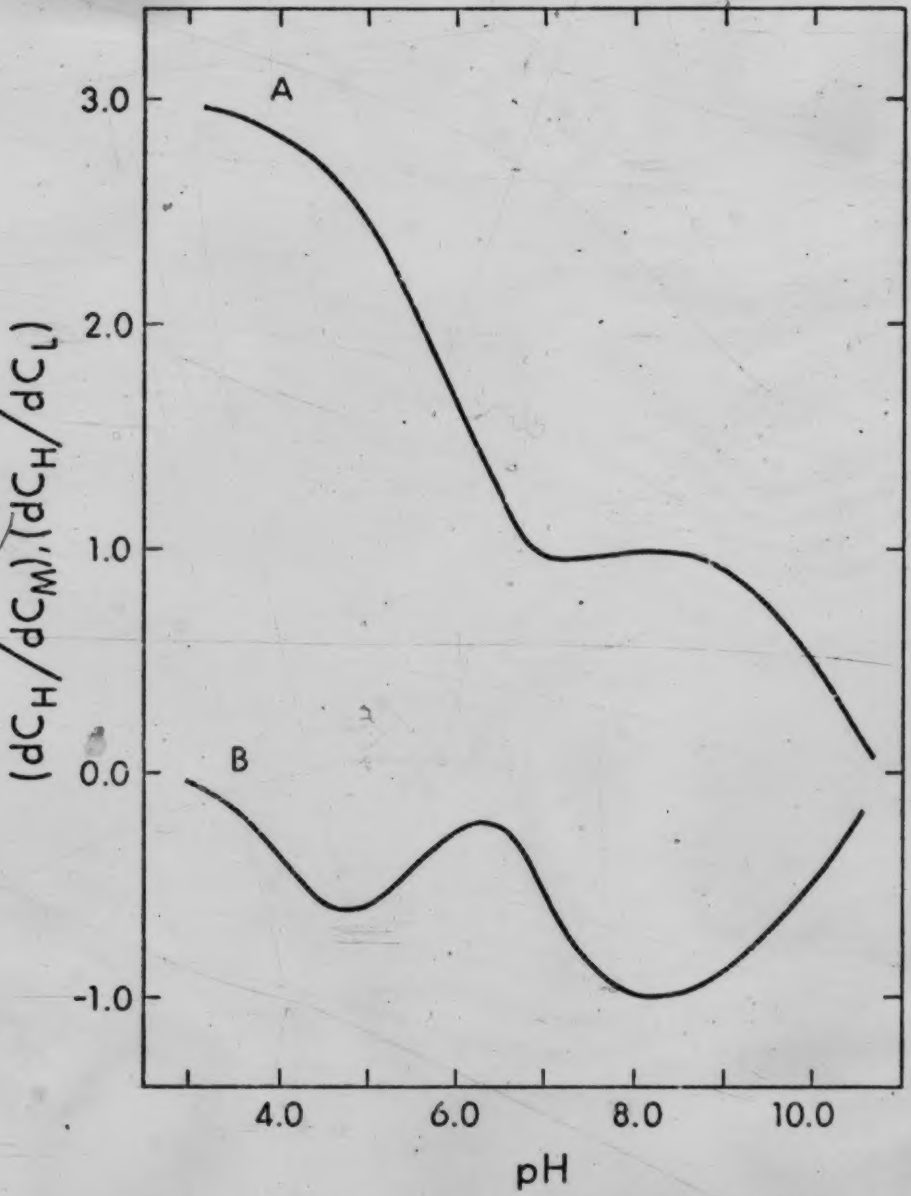


Figure 5 . $(dC_H/dC_L)_{C_M,pH}$, A; and $(dC_H/dC_M)_{C_L,pH}$, B; for a system containing a triprotic ligand and complexes H_2ML, HM_2L and M_2L_2. The overall protonation constants are 1.0×10^{10}, 1.0×10^{16} and 1.0×10^{21}. The formation constants of the complexes are 1.0×10^{19}, 1.0×10^{18} and 1.0×10^{17} respectively. $C_M = 1.0 \times 10^{-3}$ M, $C_L = 2.5 \times 10^{-3}$ M. (See Table 4 .)

is (dC_H/dC_L) and (dC_H/dC_M) for Series E (Table 4) where C_L is 2.5 x 10^{-3} and C_M is 1.0 x 10^{-3} M.

The curves shown in Figures 4 and 5 represent the derivatives of the p[L] and p[M] functions with the pH (Eqn.12).

$$\frac{dp[L]}{dpH} = \left(\frac{dC_H}{dC_L}\right)_{C_M,pH} \qquad (12)$$

A simple series manipulation of this equation leads to a relation which allows the calculation of [L] and [M] from the experimental values of (dC_H/dC_L) and (dC_H/dC_M). Integrate both sides of Equation 12 from pH_o to pH:

$$\int_{pH_o}^{pH} \left(\frac{dp[L]}{dpH}\right) dpH = \int_{pH_o}^{pH} \left(\frac{dC_H}{dC_L}\right)_{C_M,pH} dpH \qquad (13)$$

Simplify the left hand quantity.

$$\int_{pH_o}^{pH} dp[L] = \int_{pH_o}^{pH} \left(\frac{dC_H}{dC_L}\right)_{C_M,pH} dpH \qquad (14)$$

Integrate dp[L] from pH_o to pH to get $p[L]_o$ at pH_o and p[L] at pH.

$$p[L] = p[L]_o - \int_{pH_o}^{pH} \left(\frac{dC_H}{dC_L}\right)_{C_M, pH} dpH \qquad (15)$$

The integral may be evaluated in one of two ways. First , as discussed above, the entire set of (dC_H/dC_X)'s may be fit to a polynomial , and the integral evaluated by simple calculus. Second, it may be evaluated numerically using Simpson's parabolic rule, or the trapezoid rule. In this work the trapezoid rule was used to evaluate the integral directly from the tabulated $(dC_H/dC_X)_{C_Z, pH}$ function. It was found that this procedure, used at 0.1 pH increments, gave as good accuracy as is in most cases justified by the accuracy of the experimental data.

Formation Constants

The FICS method provides free ligand and free metal concentrations as a function of pH. Formation constants can be extracted from a linear least squares fit to the mass balance of each component in solution. In such a procedure, the sum of the square of the residuals, U, is minimized.

$$U = \sum \left[C_L - \left([L] + \sum q \beta_{pqr} [H]^p [L]^q [M]^r \right) \right]^2 \qquad (16)$$

In all the examples presented in this thesis a Gaussian elimination (57) followed by matrix inversion has been used to solve the simultaneous equations. The mass balances for the ligand and metal were treated individually, or in some cases together, while the mass balance for protons was not used to calculate formation constants. In this respect the FICS method has considerable advantage over other pH titration methods. Several problems are inherent to the use of C_H in the calculation of formation constants, and these are avoided with the FICS method. The free hydrogen ion concentration is generally difficult to determine experimentally, involving either an estimation of the hydrogen ion activity coefficient, γ_{H^+}, or an electrode calibration in terms of $[H^+]$. At low and high pH's $[H^+]$ and $[OH^-]$ constitute a large portion of C_H, and if there is uncertainty in their values this will produce a large uncertainty in the concentration of bound protons. In comparison however, the FICS method does not require $[H^+]$ at any stage. The electrodes may be standardized with NBS reference solutions, and an estimate of γ_{H^+} is only required if the mixed activity-concentration constants are converted to concentration constants.

For simplicity in this work, the notation $\sum L$ will be used if the constants are evaluated from the ligand mass balance, $\sum M$ if they are evaluated from the metal mass balance, and $\sum ML$ if both mass balances are used simultaneously.

Some estimate of the accuracy of the formation constants obtained by the FICS method is necessary. The simplest, though not the most reliable quantity, is the goodness of the fit of the model to the known total analytical concentrations of ligands and metals. The experimental values of [L] and [M] and the formation constants are inserted into Equation 16 and the standard deviation of the fit is $[U/(n-1)]^{1/2}$, where n is the number of experimental points. The relative standard deviation of the fit, in percent, has been shown for several examples in this work.

The standard deviation calculation is limited in that it does not give any indication of the accuracy of the [L] and [M] data, rather it only estimates the fit of the hypothesized model to C_L and C_M. A better measure of the reliability of the model and the formation constants can be had by simulating titration curves and by comparing them to the original data. This was done as a final check with the metal-glutathione complexation study reported in Chapter VI. However, it is impractical to use routinely while attempting to find a model for complexation because the simulation procedures are slow and, in addition, may run into difficulties specific to each model proposed to describe the complexation.

The suitability of a model describing the composition of complex species existing in solution may be checked in several ways. It is expected that a good hypothesis will give a low standard deviation of the fit and that the constants derived from one mass balance will agree with those derived from another. The constants which are known, for example ligand protonation constants, must be derived correctly from the data. The existence of some species may be questioned if small or negative formation constants are produced, or if the agreement of values based on different mass balances is poor.

The next section will deal with the effects of various types of errors, and the problem of assigning a model to the system.

C. The Effect of Error on the FICS Method.

This discussion will be limited to error introduced through the experimental procedure. The systems of simulated titrations discussed to this point have been designed to represent feasible experiments, but without the error associated with the actual experiment. In this section on errors, several systematic and random errors inherent to a real experiment will be considered. The error in [L] and [M] will then include uncertainty arising from both the FICS data handling and the imposed experimental error.

Dilution Error

Three separate types of error are introduced by dilution. First, during the series of titrations necessary to evaluate $(dC_H/dC_L)_{C_M,pH}$ the values of C_L are not constant but vary according to the quantity of titrant added to that pH. This is easily compensated for by using C_L exactly as it is found at that pH, based upon volume of titrant added to that point. Each pH will have a unique set of C_L's through which the derivative is evaluated. Series A and B in Table 1 were simulated including significant dilution, and the values for [L] shown in Table 6 agree very well with the expected values.

The second and third dilution problems are related to dilution of components other than C_L, and cannot be corrected in the data handling process. During a set of titrations designed to give the derivative $(dC_H/dC_L)_{C_M,pH}$, the C_M will vary through dilution. Assuming that all the titrations have equal dilution to a given pH, the derivative will be evaluated at a C_M different from C_M at pH_o.

The third type of error is implied above. All the titrations do not have equal dilution to a given pH, and consequently, at each pH, C_M will be different for the different titrations. The term $(dC_H/dC_L)_{C_M,pH}$ explicitly requires that the change in C_H with C_L be measured at constant pH and C_M.

These problems contribute significant error to the free ion concentrations. Table 8 shows [L] and [M] for Series C titrations with and without dilution. With a total dilution of 7.201 ml titrant into 300 ml, the free ligand concentration at pH 10.0 had about 10% error and the free metal concentration about 25%. Without dilution the error in [L] and [M] was only 0.5%. Table 9 summarizes formation constants derived from these two sets of free ion concentration data.

The second and third types of dilution error, discussed above, cannot be corrected once the data has been taken. On the other hand, it is possible to experimentally ensure that C_M remains constant throughout a set of titrations, either by eliminating dilution by use of coulometric titration, or by simultaneous addition of titrant and a concentrated metal solution. Computer automation of the latter is a practical alternative, and was used for the studies of complexation reported in this thesis. Details of the manner in which this was carried out experimentally are reported in Chapter IV.

Errors in C_L and C_M

The experimental values of C_L, C_M, C_H and pH and so on may have systematic and random errors associated with them. Studies with simulated titration data show that systematic errors give rise to the most serious problems with the FICS method. The nature of the calculation allows small

Table 8. The Results of the FICS Calculations on the Simulated Titration Data,

Series C and C'

pH	Titrant Volume (ml)	Free Ligand Known	Free Ligand Calculated	Free Metal Known	Free Metal Calculated
		Series C -- No Dilution			
3.0	-	2.756×10^{-15}	2.756×10^{-15}	7.839×10^{-4}	7.839×10^{-4}
4.0	-	1.863×10^{-12}	1.860×10^{-12}	5.094×10^{-5}	5.104×10^{-5}
5.0	-	9.127×10^{-10}	9.100×10^{-10}	1.002×10^{-6}	1.007×10^{-6}
6.0	-	5.443×10^{-8}	5.432×10^{-8}	2.629×10^{-8}	2.641×10^{-8}
7.0	-	9.124×10^{-7}	9.111×10^{-7}	5.974×10^{-10}	5.997×10^{-10}
8.0	-	9.805×10^{-6}	9.794×10^{-6}	9.456×10^{-12}	9.493×10^{-12}
9.0	-	9.083×10^{-5}	9.070×10^{-5}	1.200×10^{-13}	1.206×10^{-13}
10.0	-	5.000×10^{-4}	4.989×10^{-4}	3.996×10^{-15}	4.022×10^{-15}
		Series C' -- With Dilution			
3.0	0.3437	2.753×10^{-15}	2.753×10^{-15}	7.832×10^{-4}	7.839×10^{-4}
4.0	2.240	1.849×10^{-12}	1.868×10^{-12}	5.092×10^{-5}	4.833×10^{-5}
5.0	3.251	9.033×10^{-10}	9.141×10^{-10}	1.003×10^{-6}	0.8804×10^{-6}
6.0	5.458	5.355×10^{-8}	5.524×10^{-8}	2.661×10^{-8}	2.225×10^{-8}
7.0	6.300	8.937×10^{-7}	9.402×10^{-7}	6.097×10^{-10}	4.916×10^{-10}
8.0	6.682	9.591×10^{-6}	10.32×10^{-6}	9.667×10^{-13}	7.545×10^{-12}
9.0	6.818	8.881×10^{-5}	9.767×10^{-5}	1.227×10^{-13}	0.9290×10^{-13}
10.0	7.201	4.882×10^{-4}	5.474×10^{-4}	4.092×10^{-15}	3.039×10^{-15}

Table 9. A Summary of the Formation Constants Calculated by FICS from Series C and C'

	Formation Constants		
Complexes	Known	C No Dilution	C' Dilution
H_3L	1.0×10^{21}	1.0008×10^{21}	9.893×10^{20}
H_2L	1.0×10^{16}	1.0062×10^{16}	1.073×10^{16}
HL	1.0×10^{10}	9.9996×10^{9}	1.215×10^{10}
HML	1.0×10^{17}	9.984×10^{16}	1.056×10^{17}
HML_2	1.0×10^{25}	9.993×10^{24}	1.044×10^{25}
ML_2	1.0×10^{18}	9.989×10^{17}	9.647×10^{17}

errors to propagate and give rise to substantial error in the final [L] and [M]. The error increases, in most cases, from pH_o to the final pH at a rate dependent on the size of the error on a point to point basis. The magnitude of the error seldom increases, then decreases at some further pH. The FICS calculations appear to have a tendency to accumulate error even though the error appears to be random in nature.

Systematic error in quantities such as C_L and C_M may arise in two ways. First, there is an error associated with the concentration of the stock solution from which the samples are prepared and, second, an error associated with the preparation of the samples. The latter is usually considered to be a random error, but in the FICS method it will have the same effect as a systematic error. This is because the derivatives (dC_H/dC_L) are repeatedly calculated through the pH range using for example five titrations of varying C_L. If some error in (dC_H/dC_L) arises because of an error in the sample preparation, that same error will appear in (dC_H/dC_L) at every pH where the calculations are carried out. To minimize this source of error, as many titrations as possible must be performed, the samples must be prepared with utmost care, and measures must be taken to ensure as much randomization of errors in the sample preparation as possible. For all the experimental parts of this thesis, the samples were prepared in the order 3, 2, 4, 1 and 5, where 1 would be the lowest concentration of C_L

and 5 the highest. In this way, the titrations performed first were in the middle of the C_L range and errors in sample preparation had somewhat less effect on (dC_H/dC_L).

Error in pH Measurement

Unlike other pH titration methods, the FICS procedure does not require that the hydrogen ion concentration or the hydrogen ion activity be known to get accurate free ion concentrations for ligands and metals. The pH is merely a reference scale on which to first evaluate $(dC_H/dC_L)_{C_M,pH}$ and then to integrate this quantity from pH_o to the various pH values. Its numerical value is not required in any stage of the calculation of [L] and [M]. However, the pH does enter into the calculation of formation constants. If the electrodes are standardized with NBS activity references, then the formation constants derived are mixed activity-concentration constants, and if the electrodes are calibrated for concentration, the constants are concentration constants.

The FICS method requires only that the pH electrode standardization be internally consistent, and serious systematic error is avoided. Random errors in individual measurements will exist however. These will give rise to exactly the same type of error as random errors in C_H, and will therefore be discussed in the following section.

Errors in C_H

The effects of errors in C_H are quite unlike those of errors in C_L or C_M. Errors in C_H vary from data point to data point, being the combination of an error in the quantity of titrant added to that point, and an error in the pH measurement. Unlike the results of an error in C_L, where every pH is affected more or less equally, the errors in C_H will be different at every pH value in every titration. Therefore, if errors in C_L give rise to a (dC_H/dC_L) too large by 0.05 at pH 5.0, the same error will appear at pH 6.0, and so on. The errors in (dC_H/dC_L) caused by uncertainties in C_H will be different at every pH.

In order to evaluate the effects of error on the FICS procedure, random error was imposed on simulated titration data to give some qualitative idea of the order of magnitude of errors the method may endure. However, since real error may not behave exactly as assumed here, conclusions must be drawn with some care.

Two different approaches are considered. The first is random error based on the magnitude of C_H, that is, the error is larger at larger C_H. This is similar to a constant sized error in pH. The second approach is a random error of constant magnitude. This sets the effective sensitivity of the C_H measurement. As C_H becomes small it loses significance, being the difference between two large numbers, total acid and total base concentrations.

Table 10 summarizes results of the FICS method on Series D data, including the free ligand and free metal concentrations, the constants calculated using the free ligand and free metal concentrations, and the standard deviation of the fit, in percent. Random errors of 5%, 2%, 1% and 0.5% of C_H have been imposed upon the C_H data which was used to give the results in Table 10, and the effect of these errors is shown in Table 11. The free ligand and free metal concentrations are accurate only if the error was less than 0.5%. The constants calculated using the [L] and [M] values from Table 11 are summarized in Table 12. It appears that errors of 0.5% in C_H will give very unsatisfactory results. These are random errors, however, and a systematic error of 0.5% in C_H will give much worse results.

Table 13 presents results of imposition of absolute errors of various magnitudes on C_H data from Series D. The error is reported in parts per thousand of C_{H_o} where C_{H_o} is the value of C_H at pH_o. If C_{H_o} is 1.0×10^{-2} M, then the random error is 1.0×10^{-5} or less at the 1 ppt level. This means that a C_H value of 1.0×10^{-4} has only one significant figure of accuracy, and 1.0×10^{-5} has virtually none. The results indicate that even up to 4 ppt error, significant problems do not arise in free ligand and free metal concentrations. Table 14 indicates that the errors in formation constants are moderate at the 4 ppt level, and acceptable at 2 ppt.

In summary then, the two types of error imposed on C_H

Table 10. The Results of the FICS Calculations on the Simulated Titration Data, Series D

	Free Ligand		Free Metal	
pH	Known	Calculated	Known	Calculated
4.0	1.204×10^{-5}	1.202×10^{-5}	3.414×10^{-5}	3.419×10^{-5}
5.0	1.250×10^{-4}	1.246×10^{-4}	2.732×10^{-7}	2.746×10^{-7}
6.0	5.792×10^{-4}	5.732×10^{-4}	4.314×10^{-9}	4.389×10^{-9}
7.0	1.055×10^{-3}	1.044×10^{-3}	7.151×10^{-10}	7.262×10^{-10}
8.0	1.422×10^{-3}	1.415×10^{-3}	9.959×10^{-10}	1.971×10^{-10}
9.0	1.844×10^{-3}	1.836×10^{-3}	2.463×10^{-11}	2.474×10^{-11}
10.0	1.980×10^{-3}	1.973×10^{-3}	2.498×10^{-12}	2.510×10^{-12}

	Formation Constants		
Complexes	Known	Sum over C_L	Sum over C_M
H_2L	1.0×10^{10}	9.983×10^{9}	
HL	1.0×10^{6}	9.952×10^{5}	
ML	1.0×10^{6}	1.021×10^{6}	1.004×10^{6}
ML_2	1.0×10^{11}	9.978×10^{10}	9.948×10^{10}
ML_3	1.0×10^{15}	1.019×10^{15}	1.015×10^{15}
$H_{-1}ML_2$	1.0×10^{4}	1.004×10^{4}	1.002×10^{4}

Table 11. The Effects of Random Errors in C_H on the Free Ligand and Free Metal Concentrations Calculated by FICS[a]

pH	Known	±5%[b]	±2%	±1%	±0.5%
	Free Ligand				
4.0	1.204×10^{-5}	1.50[c]	1.32	1.21	1.21
5.0	1.250×10^{-4}	1.62	1.38	1.32	1.27
6.0	5.792×10^{-4}	6.80	6.14	6.00	5.86
7.0	1.055×10^{-3}	1.24	1.12	1.09	1.07
8.0	1.422×10^{-3}	1.68	1.51	1.47	1.45
9.0	1.844×10^{-3}	2.19	1.97	1.92	1.88
10.0	1.980×10^{-3}	2.42	2.14	2.06	2.01
	Free Metal				
4.0	3.414×10^{-5}	8.08	4.82	3.40	3.23
5.0	2.732×10^{-7}	6.80	3.95	2.91	2.52
6.0	4.314×10^{-9}	8.34	5.67	4.99	4.17
7.0	7.151×10^{-10}	14.20	9.50	8.27	6.95
8.0	1.959×10^{-10}	3.82	2.57	2.24	1.88
9.0	2.463×10^{-11}	4.91	3.25	2.70	2.37
10.0	2.498×10^{-12}	5.40	3.41	2.70	2.47

[a] The random error in C_H is proportional to the magnitude of C_H.

[b] The size of the random error, in percent of C_H.

[c] Same order of magnitude as known ligand concentration.

Table 12. The Effect of Random Error in C_H on the Formation Constants Calculated by FICS[a]

		Formation Constants						
		±5%[b]	±2%		±1%		±0.5%	
Complexes	**Known**	ΣL	ΣL	ΣM	ΣL	ΣM	ΣL	ΣM
H_2L	1.0×10^{10}	1.00	1.00		0.982		1.005	
HL	1.0×10^{6}	1.08	1.01		0.909		1.026	
ML	1.0×10^{6}	0.715	0.970	0.855	1.08	1.01	0.854	0.964
ML_2	1.0×10^{11}	-1.29	0.259	0.388	1.06	0.951	1.059	1.101
ML_3	1.0×10^{15}	3.21	0.652	0.674	0.753	0.755	0.982	0.985
$H_{-1}ML_2$	1.0×10^{4}	0.240	0.575	0.626	0.808	0.847	0.972	0.991
S.D. of Fit		-	2.2%	3.8%	1.1%	2.1%	0.41%	0.99%

[a] The random error in C_H is proportional to the magnitude of C_H.

[b] The size of the random error, in percent of C_H.

Table 13. The Effect of Random Error in C_H on the Free Ligand and Free Metal Concentrations Calculated by FICS [a]

	Free Ligand			
		Parts per Thousand of C_{H_o}		
pH	Known	1	2	4
4.0	1.204×10^{-5}	1.21	1.20	1.21
5.0	1.250×10^{-4}	1.25	1.25	1.23
6.0	5.792×10^{-4}	5.72	5.78	5.81
7.0	1.055×10^{-3}	1.04	1.05	1.05
8.0	1.422×10^{-3}	1.42	1.42	1.42
9.0	1.844×10^{-3}	1.84	1.85	1.85
10.0	1.980×10^{-3}	1.97	1.99	1.99
	Free Metal			
4.0	3.414×10^{-5}	3.39	3.39	3.57
5.0	2.732×10^{-7}	2.74	2.71	2.85
6.0	4.314×10^{-9}	4.34	4.22	4.47
7.0	7.151×10^{-10}	7.26	7.11	6.99
8.0	1.959×10^{-10}	1.98	1.95	1.91
9.0	2.463×10^{-11}	2.56	2.50	2.41
10.0	2.498×10^{-12}	2.57	2.52	2.44

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[a] The random error in C_H is of constant magnitude, shown in parts per thousand of C_{H_o}

Table 14. The Effect of Random Error on the Formation Constants Calculated by FICS[a]

Complexes	Exact	Formation Constants					
		Parts per Thousand of C_{H_O}					
		1 ppt		2 ppt		4 ppt	
		ΣL	ΣM	ΣL	ΣM	ΣL	ΣM
H_2L	1.0×10^{10}	0.989		0.989		1.04	
HL	1.0×10^{6}	1.05		0.951		0.734	
ML	1.0×10^{6}	1.04	1.02	1.17	1.05	0.975	0.980
ML_2	1.0×10^{11}	0.913	0.990	1.00	0.961	1.24	0.934
ML_3	1.0×10^{15}	1.03	1.02	1.03	1.03	1.02	1.02
$H_{-1}ML_2$	1.0×10^{4}	0.973	0.971	0.957	0.968	0.990	0.999
S.D. of Fit		0.53%	0.67%	0.65%	0.97%	1.6%	2.6%

[a] The random error in C_H is of constant magnitude, shown in parts per thousand of C_{H_O}.

indicate that the FICS method requires accuracy of C_H of the order of 2 ppt or less for most reliable results. It must also be kept in mind that the ligand to metal ratio, and so on, used in the Series D data simulation were chosen with all those factors in Part B of this chapter given careful consideration. These conclusions cannot be applied to situations where C_H is unnecessarily small or where there is severe curvature in the function of C_H vs C_L.

The Effects of Errors in the Initial Free Ligand and Free Metal Concentrations

Since the FICS procedure requires free ligand and free metal at some starting point, this imposes restrictions which contradict many of the experimental requirements discussed above. Free ligand can be calculated from the total ligand in the presence of metal only at low pH, where protons compete successfully for ligand. However, a large excess of protons means that C_{H_o} will be large, and C_H will therefore lose significance at correspondingly higher values, bringing about the problems discussed in an earlier section.

Table 15 illustrates the effects of error in $p[L]_o$ and $p[M]_o$ on the free ligand and free metal values derived from Series D data. The exact [L] and [M] may be found in Table 10.

The far left column in Table 15 is the set of [L] and [M] calculated using $p[L]_o$ of 6.439, $p[M]_o$ of 3.000, based

Table 15. The Effect of Systematic Error in pX_o on Free Ligand and Free Metal Concentrations Calculated by FICS

	pL_o and pM_o		
	6.439	6.459	6.466
	3.000	3.085	3.114
pH	Free Ligand		
4.0	1.29×10^{-5}	1.23×10^{-5}	1.21×10^{-5}
5.0	1.34×10^{-4}	1.27×10^{-4}	1.26×10^{-4}
6.0	6.14×10^{-4}	5.86×10^{-4}	$.577 \times 10^{-4}$
7.0	1.12×10^{-3}	1.07×10^{-3}	1.05×10^{-3}
8.0	1.52×10^{-3}	1.45×10^{-3}	1.42×10^{-3}
9.0	1.97×10^{-3}	1.88×10^{-3}	1.85×10^{-3}
10.0	2.11×10^{-3}	2.01×10^{-3}	1.99×10^{-3}
	Free Metal		
4.0	4.62×10^{-5}	3.79×10^{-5}	3.54×10^{-5}
5.0	3.71×10^{-7}	3.05×10^{-7}	2.85×10^{-7}
6.0	5.39×10^{-10}	4.87×10^{-9}	4.55×10^{-9}
7.0	9.81×10^{-10}	8.06×10^{-10}	7.53×10^{-10}
8.0	2.66×10^{-10}	2.19×10^{-10}	2.04×10^{-10}
9.0	3.34×10^{-11}	2.74×10^{-11}	2.57×10^{-11}
10.0	3.39×10^{-12}	2.78×10^{-12}	2.60×10^{-12}

Table 15 (continued)

	pL$_o$ and pM$_o$	
	6.468	6.469
	3.125	3.130
pH	Free Ligand	
4.0	1.20×10^{-5}	1.20×10^{-5}
5.0	1.25×10^{-4}	1.25×10^{-4}
6.0	5.74×10^{-4}	5.73×10^{-4}
7.0	1.05×10^{-3}	1.04×10^{-3}
8.0	1.42×10^{-3}	1.42×10^{-3}
9.0	1.84×10^{-3}	1.84×10^{-3}
10.0	1.98×10^{-3}	1.97×10^{-3}
	Free Metal	
4.0	3.46×10^{-5}	3.42×10^{-5}
5.0	2.78×10^{-7}	2.75×10^{-7}
6.0	4.44×10^{-9}	4.39×10^{-9}
7.0	7.34×10^{-10}	7.26×10^{-10}
8.0	1.99×10^{-10}	1.97×10^{-10}
9.0	2.50×10^{-11}	2.47×10^{-11}
10.0	2.54×10^{-12}	2.51×10^{-12}

Table 16. The Effect of Systematic Error in pX_o on Formation Constants Calculated by FICS

	pL_o and pM_o			
	6.439		6.459	
	3.000		3.085	
Complexes	ΣL	ΣM	ΣL	ΣM
H_2L	9.28×10^9		9.75×10^9	
HL	9.90×10^5		9.93×10^5	
ML	6.78×10^5	5.32×10^5	8.90×10^5	8.21×10^5
ML_2	6.06×10^{10}	7.02×10^{10}	8.45×10^{10}	8.84×10^{10}
ML_3	5.98×10^{14}	5.99×10^{14}	8.53×10^{14}	8.52×10^{14}
$H_{-1}ML_2$	6.01×10^3	6.48×10^3	8.47×10^3	8.65×10^3
S.D. of Fit	0.13%	3.9%	0.12%	1.2%

Table 16 (continued)

Complexes	pL$_o$ and pM$_o$			
	6.466 3.114		6.468 3.125	
	ΣL	ΣM	ΣL	ΣM
H_2L	9.90×10^9		9.96×10^9	
HL	9.94×10^5		9.95×10^5	
ML	9.73×10^5	9.37×10^5	1.01×10^6	9.83×10^5
ML_2	9.42×10^{10}	9.55×10^{10}	9.80×10^{10}	9.82×10^{10}
ML_3	9.58×10^{14}	9.55×10^{14}	1.00×10^{15}	9.96×10^{14}
$H_{-1}ML_2$	9.47×10^3	9.52×10^3	9.86×10^3	9.86×10^3
S.D. of Fit	0.12%	0.44%	0.12%	0.18%

on the assumption that no complexation of metal has occurred, while in fact about 25% of the metal is coordinated to ligands. There is consequently significant error in [L] and [M]. This problem may be corrected with the following algorithm:

(1) assume no complexation, calculate $p[L]_o$ and $p[M]_o$,

(2) calculate [L] and [M] using $p[L]_o$ and $p[M]_o$ from (1) (for example Column 1, Table 15),

(3) calculate formation constants based upon these [L] and [M] values (Column 1, Table 16); the entire set of [L] and [M] may not necessarily be used,

(4) calculate new values for $p[L]_o$ and $p[M]_o$ based on the estimates of constants derived: best estimates for protonation constants or any known constants should be used,

(5) determine a new set of [L] and [M] based upon the new $p[L]_o$ and $p[M]_o$ (Column 2, Table 16), and

(6) determine a new set of constants, and a new estimate of $p[L]_o$ and $p[M]_o$ (Column 2, Table 16).

As the results in Tables 15 and 16 show, the [L] and [M] improve, until they are more or less exact. As the $p[L]_o$ and $p[M]_o$ are improved, the constants are more reliable, and change less with each iteration.

This method will fail if too much metal or ligand are complexed at pH_o. If the types of complexes at low pH are not known, the procedure will also fail.

Values of $p[L]_o$ and $p[M]_o$ may also be found by a trial and error approach in which the standard deviation of the fit is minimized. Each $p[L]_o$ and $p[M]_o$ is used to get a set of formation constants and these constants, with the [L] and [M] data are fit to the total ligand and metal mass balances. The residual defined by Equation 16 will reach a minimum when $p[L]_o$ and $p[M]_o$ provide a set of [L] and [M] most closely matching that of the model of complexation. This procedure will not be meaningful if the model for complexation is incorrect. This method has the advantage that it choses the best $p[L]_o$ and $p[M]_o$ in terms of the entire range of pH, but the disadvantage that a model of complexation over a wide range of pH must be known. Both of these approaches have been used with experimental data and give good results.

D. Determination of a Model for Metal-Ligand Interactions.

Titration methods do not in general provide information from which the model, or the types of complexes in a system, can be derived. In this respect, FICS has an advantage over other methods, in that the FICS results can be used to provide some guidance in the selection of a model. Using [H], [L] and [M], the concentrations of proton, ligand and metal whose chemical situation has not been determined (ΔC_H, ΔC_L and ΔC_M) can be calculated.

For example, given the total ligand concentration C_L, and the free ligand concentration [L], ΔC_L equals (C_L - [L]). If the pH and the protonation constants are known,

$$\Delta C_L = C_L - ([L] + \beta_1[H][L] + \beta_2[H]^2[L]) \qquad (17)$$

If the formation constant for HML is known:

$$\Delta C_L = C_L - ([L] + \beta_1[H][L] + \beta_2[H]^2[L] + \beta_3[H][L][M]) \qquad (18)$$

Figures 6 and 7 are combined plots of ΔC_H, ΔC_L and ΔC_M for the Series E data (see Table 4). These quantities were calculated from C_H, C_L, C_M and the pH, [L] and [M] from Table 17. Three distinct areas can be seen:

(1) below pH 3.5 there is a region where ΔC_H is about twice as large as ΔC_L or ΔC_M. The plot indicates that this complex reaches a concentration of about 1.0×10^{-4} M, and then is replaced by another appearing in larger quantities.

(2) the predominant complexes near pH 6.0 are composed of more metal than protons or ligands. The ratio of metal to protons or ligands is about two to one.

(3) above pH 6.0 the complexes which predominate contain only metal and ligands, in equal proportions, perhaps ML or M_2L_2.

Figure 7 is an expanded part of Figure 6 and shows that the first complex to form at low pH must be H_2ML. Using the [L] and [M] data from pH 3 to 5 the formation constant for this complex was found to be 4.0×10^{18}.

The ΔC_H, ΔC_L and ΔC_M values were calculated using this

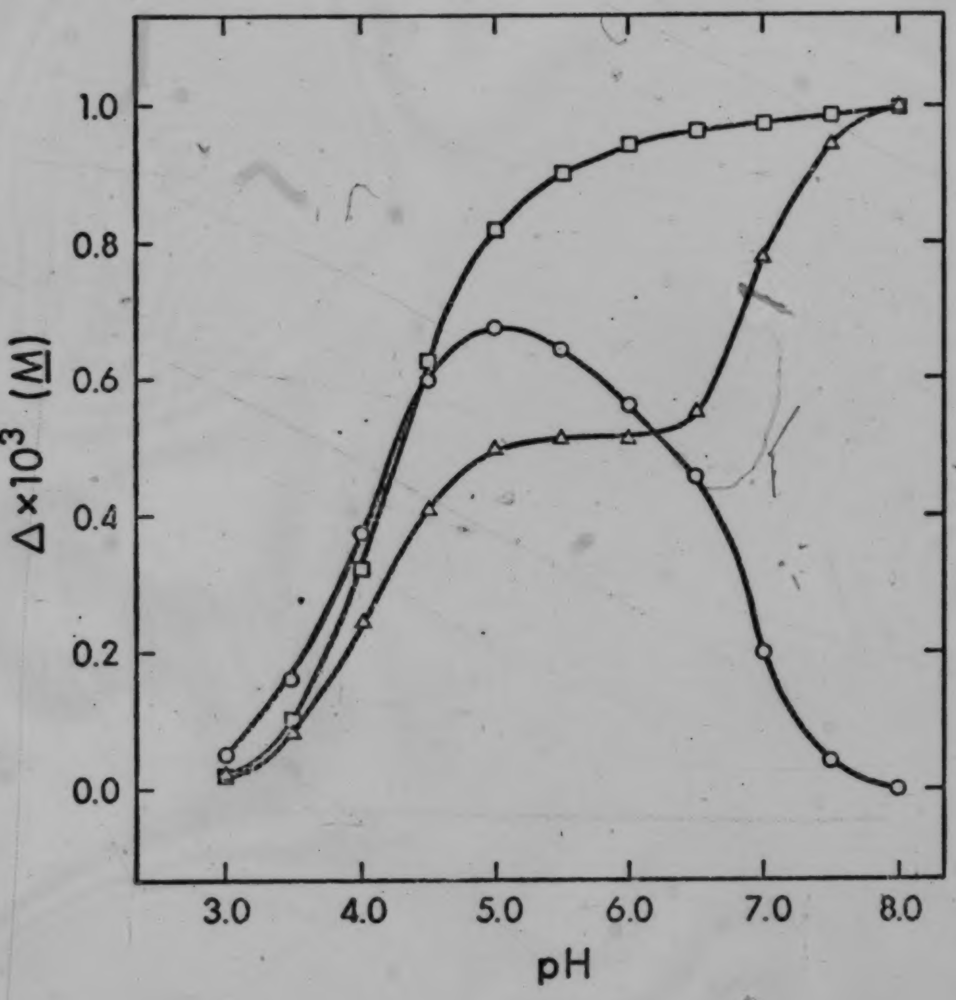


Figure 6 . ΔC_H, ○; ΔC_L, △ ; and ΔC_M, □ ; for the system of complexes defined in Figure 5 . These quantities were calculated directly from C_H, C_L and C_M, and the pH, [L] and [M] values in Table 17.

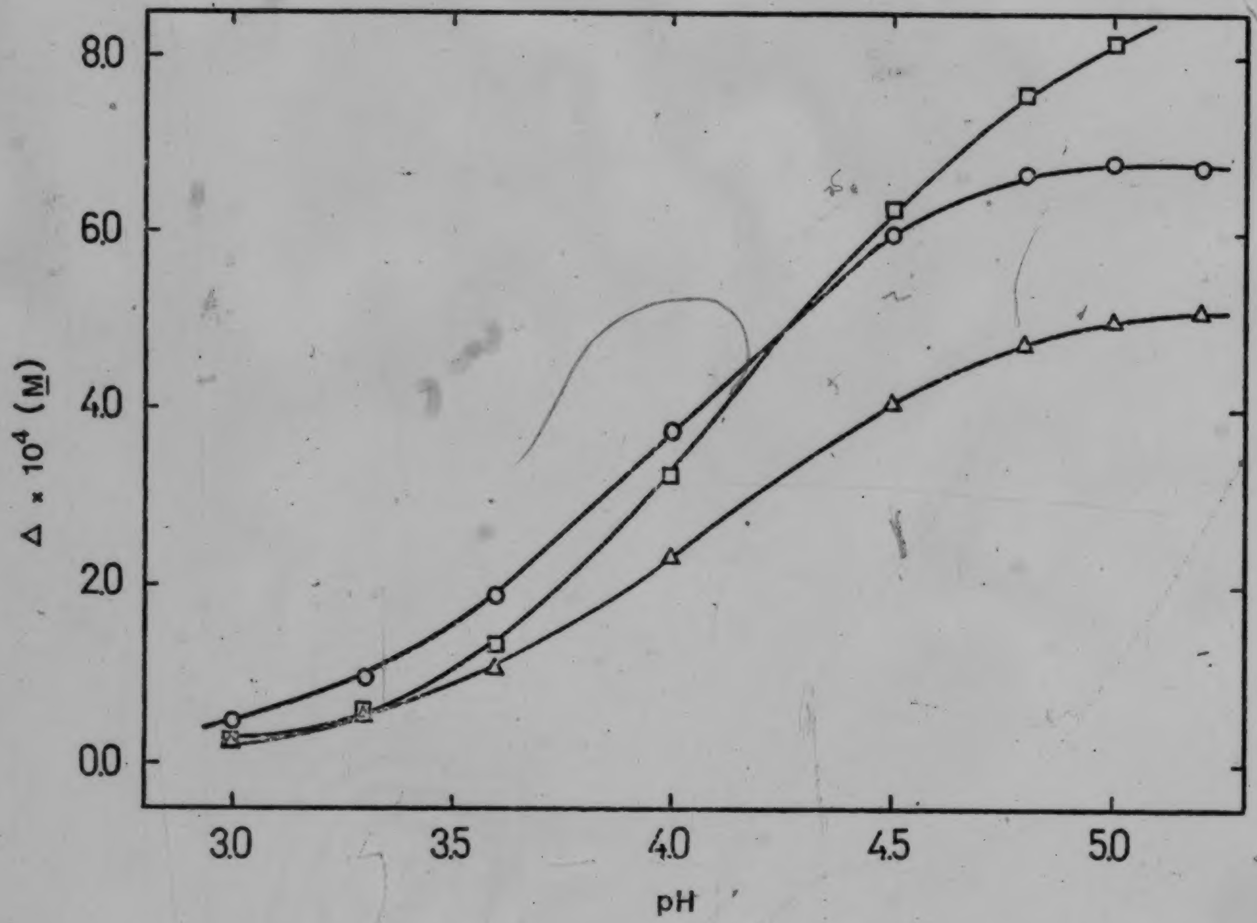


Figure 7 . ΔC_H, ○; ΔC_L, △ ; and ΔC_M, □ ; as in Figure 6 . The low pH region is shown in more detail.

Table 17. The Results of the FICS Calculations on the Simulated Titration Data, Series E

pH	Free Ligand		Free Metal	
	Known	Calculated	Known	Calculated
4.0	2.060×10^{-12}	2.059×10^{-12}	6.740×10^{-4}	6.737×10^{-4}
5.0	9.544×10^{-10}	9.514×10^{-10}	1.834×10^{-4}	1.838×10^{-4}
6.0	9.511×10^{-8}	9.474×10^{-8}	6.726×10^{-5}	6.749×10^{-5}
7.0	1.563×10^{-6}	1.557×10^{-6}	3.462×10^{-5}	3.467×10^{-5}
8.0	1.478×10^{-5}	1.465×10^{-5}	4.755×10^{-6}	4.780×10^{-6}
9.0	1.363×10^{-4}	1.349×10^{-4}	5.187×10^{-7}	5.217×10^{-7}
10.0	7.500×10^{-4}	7.419×10^{-4}	9.427×10^{-8}	9.490×10^{-8}

Complexes	Formation Constants		
	Known	Sum over C_L	Sum over C_M
H_3L	1.0×10^{21}	1.000×10^{21}	
H_2L	1.0×10^{16}	1.000×10^{16}	
HL	1.0×10^{10}	9.921×10^{9}	
H_2ML	1.0×10^{19}	9.748×10^{18}	1.005×10^{19}
HM_2L	1.0×10^{18}	1.034×10^{18}	9.966×10^{17}
M_2L_2	1.0×10^{17}	1.033×10^{17}	1.008×10^{17}
S.D. of Fit		0.07%	0.06%

estimated constant. The ΔC_L plots are shown in Figure 8, for several guesses of that constant. The first guess, 4.0×10^{18}, was too low, since ΔC_L remained large. A guess of 2.6×10^{19} proved to be too large, giving negative ΔC_L's. The guess of 1.5×10^{19} was also too large. The value 1.2×10^{19} was good, giving a ΔC_L of nearly zero from pH 3.0 to 3.5.

Figure 9 is a combined plot of ΔC_H, ΔC_L and ΔC_M using the initial guess of 1.2×10^{19} for H_2ML. The complex near pH 5.0 is almost exclusively of a HM_2L type stoichiometry. The free ligand and free metal data up to pH 6.0 was used to get estimates of formation constants for the two complexes, H_2ML and HM_2L. These were found to be about 1.02×10^{19} and 9.98×10^{17} respectively.

Once again a combined plot of ΔC_L, ΔC_L, and ΔC_M was prepared using the estimated constants for H_2ML and HM_2L. The curves are shown in Figure 10. The estimates were very good, giving small ΔC_H, ΔC_L and ΔC_M values up to pH 6.0. The final complexes clearly contained no protons, and were composed of equal amounts of metal and ligand. The free ligand and free metal data for the entire pH range was used to get formation constants based upon the model H_2ML, HM_2L and M_2L_2 (see Table 17).

Refinement of the Model

Given that some model of the system is proposed, the

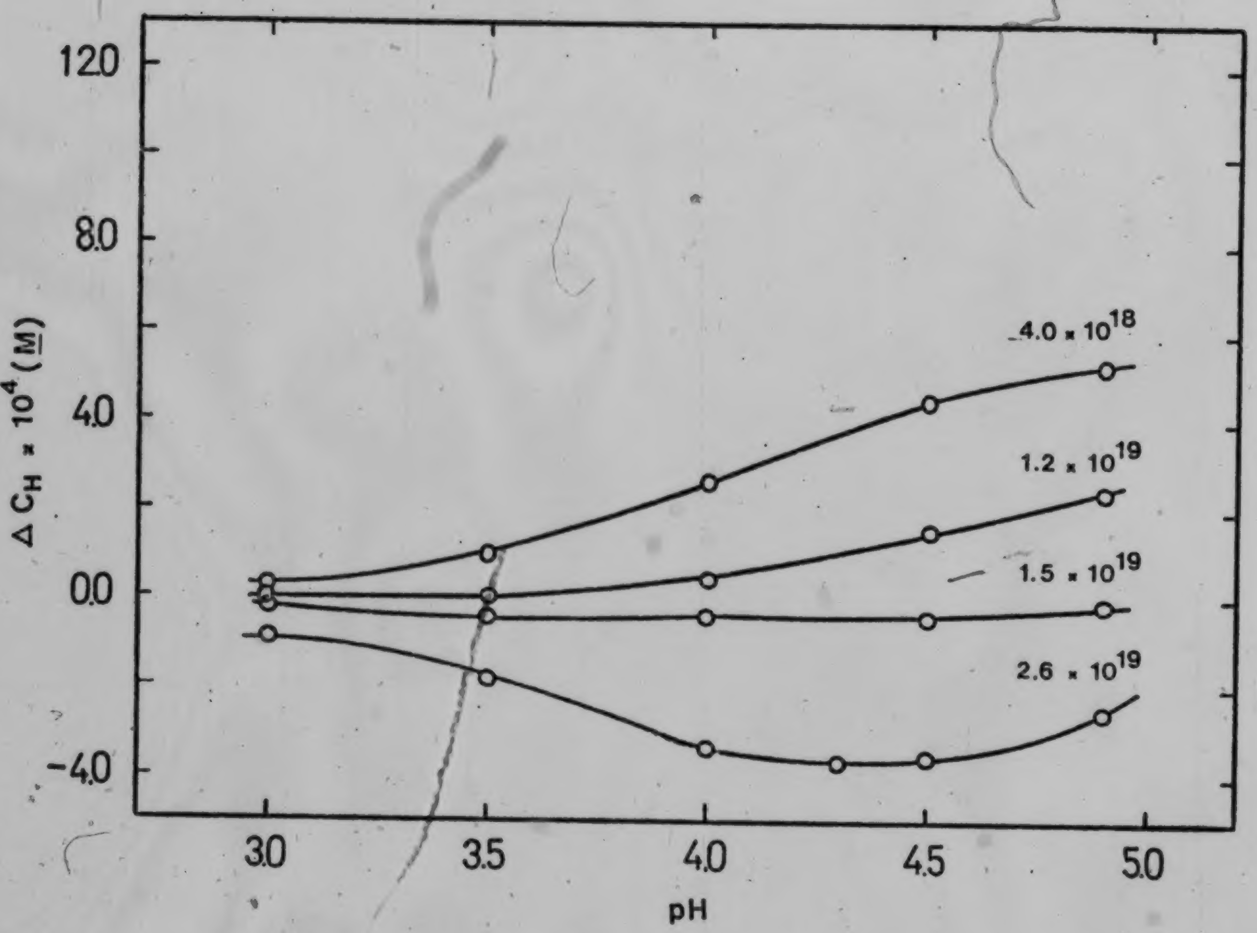


Figure 8 . ΔC_H,o; as in Figure 6 , except that the complex H_2ML is accounted for in the calculation. ΔC_H curves based on several estimates of β_{H_2ML} are shown.

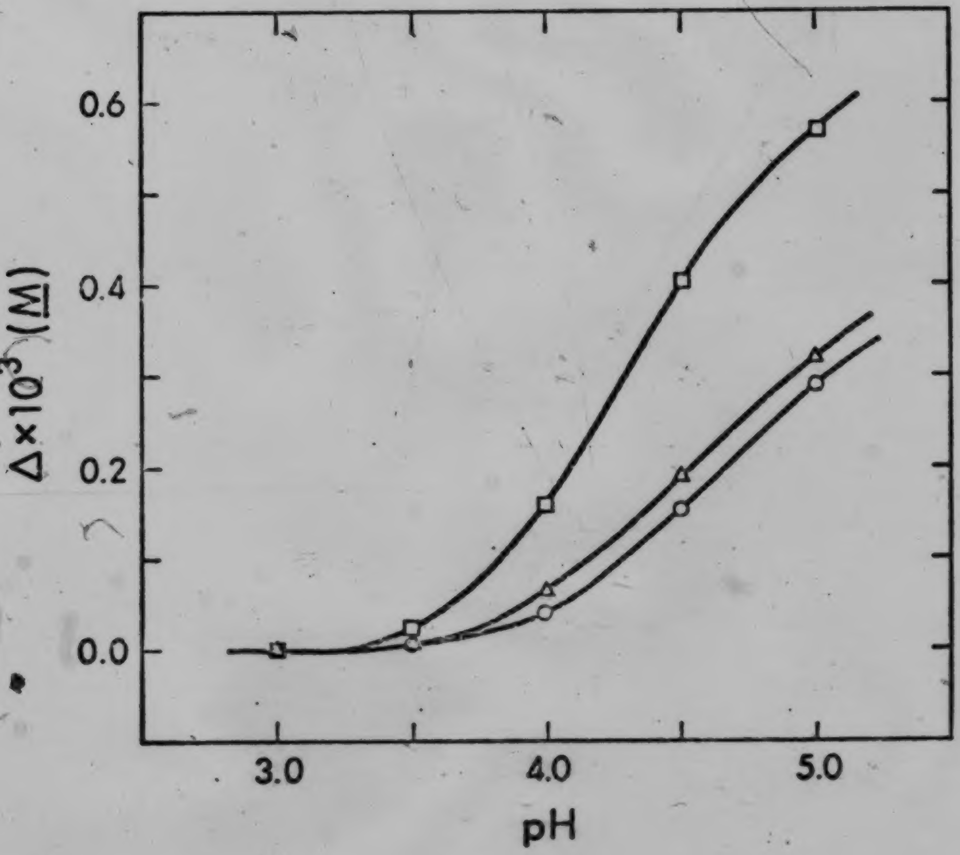


Figure 9. ΔC_H, o; ΔC_L, ▵; and ΔC_M, ▫; as in Figure 6, except that the complex H_2ML is accounted for in the calculation of ΔC_H, ΔC_L, and ΔC_M. An estimated value of 1.2×10^{19} for β_{H_2ML} was used in the calculation.

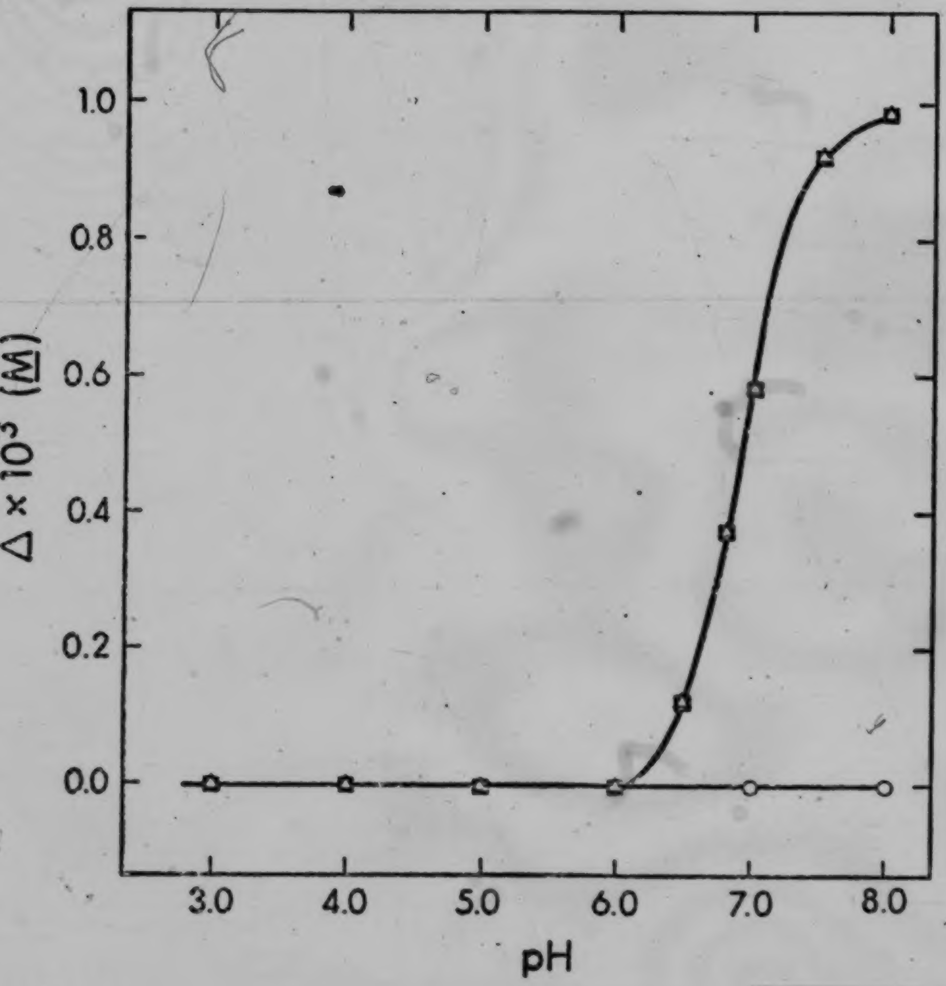


Figure 10. ΔC_H, o; ΔC_L, ▵ ; and ΔC_M, ▫ ; as in Figure 6 , except that the complexes H_2ML and HM_2L are accounted for in the calculation of ΔC_H, ΔC_L and ΔC_M. Estimated values of 1.02×10^{19} and 9.98×10^{17} were used for β_{H_2ML} and β_{HM_2L}.

final procedure must determine the minor constituents in solution and distinguish among species having the same stoichiometry. Series E, discussed above, will be used as an example.

Table 18 summarizes formation constants derived from the same [L] and [M] set, based upon the Series E. Column I is a model based on H_2ML, HM_2L and M_2L_2, and Columns II through VI are similar except one or more additional species have been included in attempts to determine which, if any, minor constituents exist in solution.

Column II includes ML, with the standard deviation of the fit somewhat improved. However, the formation constant for ML is small, with the polynuclear M_2L_2 present in large excess. In addition, the formation constants for ML based on the mass balance over C_L ($\sum L$) and over C_M ($\sum M$) are not in as good agreement as for the other species. These considerations suggest that if ML exists in solution, it is in small quantities and the constant has low reliability.

Column III is for a model which includes homologs of H_2ML, such as HML and H_3ML. The constant for H_3ML is negative in the $\sum M$ evaluation. The constants for HML are not very consistent, based upon the $\sum L$ and $\sum M$. In addition, standard deviations are not improved. In Column IV the species H_3ML was rejected, and HML remains. The constants disagree, and no improvement in the standard deviation has resulted.

Column V includes the complex M_2L, and Column VI

Table 18. Formation Constants from Free Ligand and Free Metal Data for Different Models

	I		II	
Complexes	ΣL	ΣM	ΣL	ΣM
H_3L	1.00×10^{21}		1.00×10^{21}	
H_2L	9.967×10^{15}		1.00×10^{16}	
HL	9.972×10^{9}		9.966×10^{9}	
ML			2.089×10^{5}	2.716×10^{5}
HML				
H_2ML	9.928×10^{18}	9.983×10^{18}	9.961×10^{18}	1.010×10^{19}
H_3ML				
M_2L				
HM_2L	1.031×10^{18}	1.004×10^{18}	1.020×10^{18}	1.000×10^{18}
M_2L_2	1.012×10^{17}	1.005×10^{17}	9.975×10^{16}	9.858×10^{16}
HM_2L_2				
S.D. of Fit	0.018%	0.093%	0.016%	0.030%

Table 18 (continued)

Complexes	III		IV	
	ΣL	ΣM	ΣL	ΣM
H_3L	9.863×10^{20}		1.000×10^{21}	
H_2L	9.917×10^{15}		9.972×10^{15}	
HL	9.963×10^{9}		9.964×10^{9}	
ML				
HML	2.716×10^{11}	1.05×10^{12}	3.694×10^{11}	9.544×10^{11}
H_2ML	1.085×10^{19}	1.020×10^{19}	9.975×10^{18}	1.017×10^{19}
H_3ML	1.377×10^{22}	-2.100×10^{20}		
M_2L				
HM_2L	1.029×10^{18}	9.944×10^{17}	1.025×10^{18}	9.954×10^{17}
M_2L_2	1.013×10^{17}	1.005×10^{17}	1.013×10^{17}	1.005×10^{17}
HM_2L_2				
S.D. of Fit	0.032%	-	0.017%	0.050%

Table 18 (continued)

Complexes	V		VI	
	ΣL	ΣM	ΣL	ΣM
H_3L	1.00×10^{21}		1.00×10^{21}	
H_2L	9.971×10^{15}		9.972×10^{15}	
HL	9.967×10^{9}		9.967×10^{9}	
ML				
HML				
H_2ML	9.919×10^{18}	1.006×10^{19}	9.917×10^{18}	1.005×10^{19}
H_3ML				
M_2L	4.597×10^{8}	9.162×10^{8}		
HM_2L	1.030×10^{18}	1.002×10^{18}	1.030×10^{18}	1.002×10^{18}
M_2L_2	1.012×10^{17}	1.005×10^{17}	1.012×10^{17}	1.005×10^{17}
HM_2L_2			1.540×10^{21}	5.765×10^{21}
S.D. of Fit	0.018%	0.045%	0.017%	0.042%

includes HM_2L_2. Both of these complexes are rejected. The best model in terms of standard deviation is that of H_2ML, HM_2L, ML and M_2L_2. As discussed above, the existence of ML cannot be conclusively proven.

The original Series E data did not contain the species ML. This illustrates the possible consequences of assuming that no error exists in the data. The simulated titrations did not themselves contain errors, however, slight curvature in the function of C_H vs C_L and so on have given rise to a small, but finite error in the [L] and [M] values. The only defence against this problem is good understanding of the FICS method and of the pitfalls inherent to its application to real data.

E. Summary.

The FICS method has several important advantages over most pH titration methods for the study of complex equilibria. The method provides free ion concentrations directly from the experiment. Moreover, they are derived for each component individually, that is, independent of the other components,and independent of the numerical value of the hydrogen ion concentration. In contrast to most other methods, FICS derives free ligand and free metal concentrations completely independent of any preconceived model of interactions which may be occurring in solution. Estimates of [L] and [M] have been determined even if a model for the system is never found.

The FICS procedure was tested extensively on simulated titration data, and found to be applicable to systems containing a variety of species. Examples include protonated complexes (Table 9), hydroxy complexes (Table 10), polynuclear complexes (Table 17) and mixed ligand complexes (Table 19). At this time no systems have been found for which the FICS method cannot be used in exactly the manner described in this work. No modifications of any kind, based on types or number of complexes or magnitudes of formation constants, were needed.

No attempt has been made to show that FICS will give completely exact [L] and [M]; rather, attention has been focused on each step of the procedure to find the most practical and reliable experimental method to use with FICS. In this respect it was found that situations where C_H is small and where the curvature of C_H vs C_L is large must be avoided. Systems containing an excess of ligand appear to be most favourable. Under these conditions a series of relatively simple manipulations of the experimental data will give accurate free ion concentrations.

The availability of free ligand and free metal concentrations presents excellent possibilities for the logical deduction of the complexes existing in solution. For this purpose, the quantities ΔC_H, ΔC_L and ΔC_M were introduced and applied to the deduction of a model from simulated titration data. Considerable information

Table 19. The Results of FICS Calculations on the Simulated Titration Data, Series F

	Free Ligand L		
pH	Exact	Calculated[a]	Calculated[b]
4.0	9.892×10^{-12}	9.88×10^{-12}	9.88×10^{-12}
5.0	1.150×10^{-9}	1.15×10^{-9}	1.15×10^{-9}
6.0	5.367×10^{-8}	5.26×10^{-8}	5.31×10^{-8}
7.0	9.490×10^{-7}	9.24×10^{-7}	9.37×10^{-7}
8.0	1.004×10^{-5}	9.73×10^{-6}	9.90×10^{-6}
9.0	9.127×10^{-5}	8.78×10^{-5}	8.97×10^{-5}
10.0	5.005×10^{-4}	4.80×10^{-4}	4.91×10^{-4}
	Free Ligand L'		
4.0	1.986×10^{-10}	1.99×10^{-10}	1.99×10^{-10}
5.0	1.457×10^{-8}	1.46×10^{-8}	1.46×10^{-8}
6.0	9.860×10^{-7}	9.67×10^{-7}	9.77×10^{-7}
7.0	4.846×10^{-5}	4.68×10^{-5}	4.76×10^{-5}
8.0	4.810×10^{-4}	4.63×10^{-4}	4.72×10^{-4}
9.0	9.171×10^{-4}	8.84×10^{-4}	9.02×10^{-4}
10.0	9.998×10^{-4}	9.64×10^{-4}	9.83×10^{-4}
	Free Metal		
4.0	9.787×10^{-4}	9.78×10^{-4}	9.78×10^{-4}
5.0	2.195×10^{-4}	2.18×10^{-4}	2.18×10^{-4}
6.0	1.566×10^{-6}	1.58×10^{-6}	1.57×10^{-6}
7.0	1.853×10^{-8}	1.89×10^{-8}	1.87×10^{-8}
8.0	1.000×10^{-9}	1.02×10^{-9}	1.01×10^{-9}
9.0	1.070×10^{-10}	1.09×10^{-10}	1.08×10^{-10}
10.0	1.957×10^{-11}	2.00×10^{-11}	1.9[illegible] 10^{-11}

[a] $C_{L'}$, C_L and C_M are each varied through 7 concentrations. The parameters used in these simulations are shown in Table 5.

[b] Same as above except that only the middle 5 titrations are used. This narrows the range of C_X and reduces error due to curvature in the C_H vs C_X function.

concerning the model is also potentially available from plots such as shown in Figures 1-5.

The effect of systematic and random errors of various kinds has been considered. A study of the effect of dilution revealed that significant error can result due to the systematic violation of theoretical requirements of the FICS method. The effect of error on $p[L]_o$ and $p[M]_o$ was considered, and a method suggested which will make calculation of their values possible even when a small and unknown amount of complexation has occurred. Random errors of various magnitudes were applied to titration data, and FICS found to be reliable with up to about 2 ppt random error in C_H.

The major advantage of the FICS method over other pH titration methods is that it provides an experimental value for [L] and [M] which, in turn, aids in the selection of a model and considerably reduces the possibility of having to choose among equivalent models. The major disadvantages of the method are the stringent requirements placed on the data aquisition, including its accuracy and problems involving dilution. The FICS method may not provide the best possible values for formation constants, but with good data the model of complexation can be deduced with some confidence.

Chapters III and IV will describe the methods and apparatus designed specifically for the collection of high precision data suitable for use with the FICS data handling methods.

CHAPTER III

EXPERIMENTAL

A. Materials.

All solutions were prepared with oxygen and carbon dioxide free distilled water. Removal of the gases was accomplished either by vigorous boiling, or, if the solution was acidic, by bubbling with CO_2 and O_2 free nitrogen. Boiled solutions were cooled in the CO_2 and O_2 free atomosphere of a glove box. The ionic strength of the solutions was adjusted to 0.3 moles/kg of solution with sodium perchlorate (G. Fredrick Smith Chemical Company, Columbus, Ohio).

Carbonate-free sodium hydroxide was prepared by dilution of a saturated NaOH solution (AnalaR analytical reagent NaOH, BDH Chemicals, Toronto, Ont.). The saturated NaOH was stored a minimum of one month to allow the fine particles of Na_2CO_3 to settle. All alkaline solutions were stored in, and dispensed from,polyethylene containers in a CO_2 and O_2 free atmosphere of a glove box.

Potassium hydrogen phthalate (Primary Standard, J.T. Baker Chemical Co.) was dried one hour at 120°C and cooled before sample preparation. Individual samples for titration were weighed out by difference on an analytical balance (Gram-atic, Mettler Co., Zurich, Switzerland). Sufficient quantities were used to ensure a precision of 2 parts per ten thousand.

Nitric acid solutions were prepared by dilution of the concentrated acid (J.T. Baker Chemical Co.). Individual samples for titration were weighed out by difference using a syringe and an analytical balance.

Aspartic acid was recrystallized from cold ethanolic solution, washed thoroughly with ethanol and dried in air. A stock solution was prepared by dissolving the crystals and was standardized by titration with NaOH.

Glutathione was recrystallized from a cold 50% ethanol solution, washed thoroughly with absolute ethanol and dried in air. The crystals were refrigerated until use. A short time before the experiments, a stock solution was prepared by dissolution of the crystals. This stock solution was stored under argon and in ice at all times. The recrystallization and these other precautions were taken in order to minimize the concentration of oxidized glutathione in the sample solutions. The glutathione concentration was determined by titration with NaOH, Tables 20, 21 and 22.

Stock solutions of zinc, cadmium, and lead were prepared by dissolution of the metals in HNO_3 with subsequent neutralization of the excess acid by carbonate free NaOH. These steps were carried out with small volumes in order that the final contribution of nitrate and excess acid to the sample solutions was negligible (approximately 0.005 moles/kg of nitrate and 1.0×10^{-6} moles/kg of excess

Table 20. Standardization of Sodium Hydroxide used in all the Metal-Glutathione Complexation Experiments.

Titration	Weight KHP, gm	Consumption of NaOH, gm	Calculated NaOH Concentration moles/kg
1	0.3679	5.667	0.3179
2	0.3816	5.878	0.3179
3	0.4377	6.751	0.3175
4	0.3184	4.911	0.3174
5	0.2742	4.230	0.3174
		Average	0.3176
		Relative Standard Deviation	0.08%

Table 21. Standardization of Glutathione for the Zinc-Glutathione Experiments.

Sample	Weight of Glutathione,[a] gm	Weight of Solvent,[b] gm	Consumed Weight of NaOH, gm	Concentration of Glutathione moles/kg
1	10.002	190.002	3.105	0.09640
2	8.000	192.003	2.500	0.09647
3	4.000	195.998	1.284	0.09623
4	11.001	189.002	3.405	0.09631
5	6.003	194.004	1.892	0.09640
			Average	0.09636
			Relative Standard Deviation	0.10%

[a] Stock solution

[b] Solvent contained 1.159 x 10^{-4} moles/kg of HNO_3.

Table 22. Standardization of Glutathione for the Cadmium-Glutathione and the Lead-Glutathione Experiments.

Sample	Weight of Glutathione, gm	Weight of Solvent,[a] gm	Consumed Weight of NaOH, gm	Concentration of Glutathione moles/kg
1	8.440	187.162	2.426	0.08678
2	7.432	183.485	2.146	0.08671
3	7.646	177.956	2.204	0.08681
4	8.053	199.153	2.327	0.08676
5	8.345	180.094	2.393	0.08670
			Average	0.08675
			Relative Standard Deviation	0.05%

[a] The Solvent contained 1.159 x 10^{-4} moles/kg of HNO_3.

acid). Sodium perchlorate was added to bring the ionic strength to 0.3 moles/kg. A solution density of 1021.8 gm/litre (58,59) was used to convert metal concentrations from moles/litre to moles/kg of solution.

A solution which served as a diluent, or'solvent',in the preparation of samples was composed of 0.3 moles/kg $NaClO_4$ and a small excess of nitric acid. This excess was determined by titration with NaOH. The solvent contained a slight quantity of nitric acid in order that it might serve as a pH standard at the beginning of each titration. This will be considered in more detail in the section on the experimental method.

The solutions used to compensate for dilution by titrant during the course of each titration were aliquots of the stock solutions used to prepare the samples. The samples, in general, contained about 10 gm of the stock solution of each component in a total of 200 gm. The quantity needed to compensate for each addition of titrant was therefore about 1/20 of the weight of added titrant. A titration consuming 5 gm of titrant therefore required an addition of 0.25 gm of the stock solution of that component to maintain it at constant concentration.

All of the solutions, with the exception of the zinc, cadmium and lead, were prepared and standardized on the basis of weight. The concentrations C_L, C_M, [L], [M] and so on, discussed in later sections, are reported in terms of moles/kg of solution.

B. Titration Apparatus.

The FICS method requires a considerable quantity of highly reliable data. In addition, to satisfy the theoretical requirements of the method, C_M must be constant while evaluating $(dC_H/dC_L)_{C_M,pH}$ and C_L must be constant while calculating $(dC_H/dC_M)_{C_L,pH}$. This has been done by adding appropriate quantities of concentrated components M and L to compensate for dilution caused by the titrant.

The system designed and constructed for this thesis work is shown schematically in Figure 11. It included a PDP-11/10 computer, a Fisher 520 digital pH meter, a Sartorius 3015 electronic balance and its related titrant delivery unit, and an auxiliary delivery unit composed of a Mettler DV11 digital piston drive and 1 ml buret for adding concentrated metal or ligand and a multiplexer. The PDP-11/10 computer had cassette tape and floppy disc options for storage of data and a Laboratory Peripheral System (LPS) option to facilitate input/output (I/O) operations. The parts of the LPS used in this work were the LPSDR option consisting of a sixteen bit buffered digital I/O, and the LPSKW option including a real time clock and Schmitt triggers.

Titrations were performed under computer control, and included the following operations: delivery of titrant from a gravimetric buret located on the pan of the Sartorius 3015 balance, delivery of concentrated reagents from the

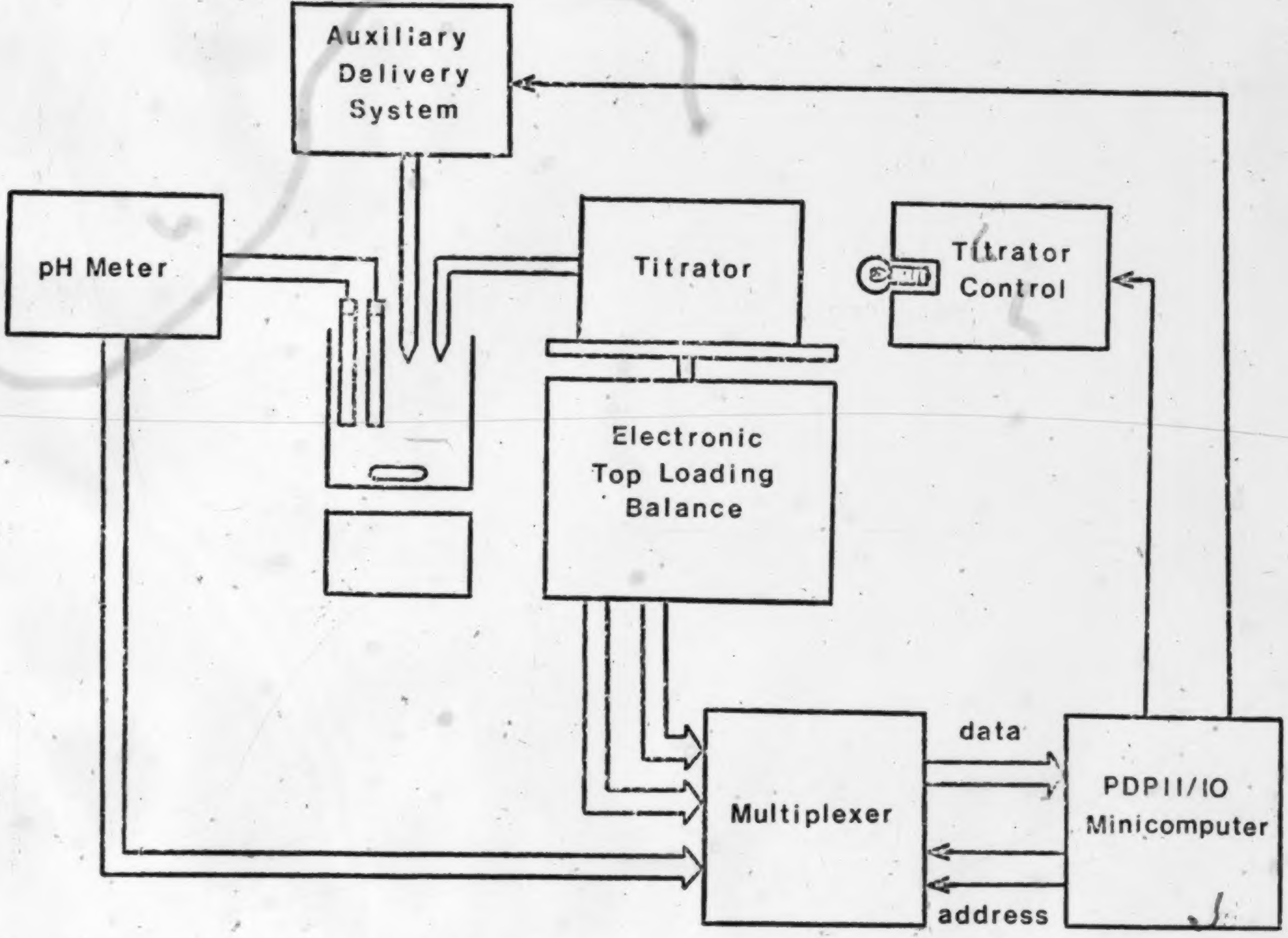

Auxiliary Delivery System
pH Meter
Titrator
Titrator Control
Electronic Top Loading Balance
Multiplexer
data
address
PDP11/10 Minicomputer

Figure 11. A Schematic Diagram of the Automatic Titration System.

METTLER DV11 auxiliary buret, sampling the digital outputs of the pH meter and electronic balance through the multiplexer, and manipulation and storage of the data for further use with the FICS method. A titration suitable for the FICS method would include a sequence of operations beginning with weighing of the unit on the balance, delivery of a portion of titrant, and reweighing. The weight of titrant delivered was used to calculate the amount of reagent to be added from the auxiliary buret, and the delivery was then executed. The electrode potential was measured and tested for equilibrium. When the electrodes had reached equilibrium, the potential was recorded and converted to pH on the basis of a calibration with NBS standards. This completed the operations required to collect a single data point, and the process was repeated every 0.1 pH unit to the end of the titration. Each data point consisted of the following information: the total weight of titrant delivered to that point, the total weight of reagents added through the auxiliary delivery unit, the equilibrium electrode potential after those additions, the pH calculated from the electrode potential and the total length of time since the titration began. This information was printed out for permanent record and stored on floppy disc for future manipulation by the FICS method.

Complete descriptions of the gravimetric titration system and details of the software control of each of the operations described above appears in Chapter IV. In the next section the method used to perform the titrations designed for the FICS calculations is described.

C. Method.

The titrations were performed at 25.0 ± 0.1 °C under an inert atmosphere of CO_2 and O_2 free nitrogen or argon. The room temperature was maintained at 23 ± 1 °C and the titrations were performed in a room having constant artificial illumination. The titration procedure consisted of delivering 0.3 moles/kg NaOH titrant to 200 gm of sample solution with a gravimetric buret. The pH of the solution was measured with a Corning glass electrode and a Ag/AgCl, Na_2SO_4 (saturated) reference electrode. Addition of concentrated reagents, as required by the FICS method, was carried out with a digital piston buret.

The glass electrode was standardized in terms of hydrogen ion activity with phthalate (pH 4.008), phosphate (pH 7.413) and carbonate (pH 10.012) NBS reference standards. This consisted of measuring the electrode potential in each solution and calculating a least squares linear regression between pH and electrode potential (mV). (See program BUFFER, Appendix C.) The electrode potential at each experimental point was converted to pH with the relation

$$pH = slope(mV) + intercept \tag{19}$$

where the slope and intercept are defined by the linear least squares fit to the NBS standards.

The solvent containing a slight excess of nitric acid served as a secondary pH standard. The hydrogen ion activity of this solution was measured in terms of the NBS standards and was used as a reference starting point for all of the metal-ligand titrations. A weighed portion of the solvent was added to a titration cell and brought to 25.0 °C, and the electrode potential was measured. The other components were then added by weight to make up the total weight of 200 gm. This eliminated electrode handling between the standardization and the beginning of each titration, an especially important consideration when using the FICS method. As described in Chapter II, the pH serves as a reference scale for the comparison of C_H from titration to titration. It is essential that this scale be consistent, especially at low pH. The solvent, containing about 10^{-4} moles/kg of HNO_3, made this possible. The pH of this solvent was assumed to be constant and provided a fixed initial point from which to start each titration, without any electrode handling between standardization with the solvent and addition of the components of the sample. The solvent was, therefore, in effect simultaneously a single point pH standard and a part of the sample solution.

As discussed in part C, Chapter II, as much care as possible must be taken to avoid systematic error in the preparation of sample solutions. Since it seemed likely that errors would be largest in the first solution prepared, the order of the titrations was chosen so that these errors would have a minimum effect on (dC_H/dC_L) and (dC_H/dC_M). The middle titrations were performed first, and those at the extremes of C_L and C_M last. The usual order was $C_L(3)$, $C_L(2)$, $C_L(4)$, $C_L(1)$ and $C_L(5)$ where $C_L(1) < C_L(2) < C_L(3) < C_L(4) < C_L(5)$.

D. Activity Coefficients.

The hydrogen ion activity served as a reference scale for use with the FICS method in the work described in this thesis. As described in the previous section, the pH of data points was calculated on the basis of the activity values of NBS standard solutions. In this way, all experimental values of [L] and [M] were measured at p_aH values. The stablity constants reported in this work are therefore mixed concentration-activity constants. Because the mass balance for C_H was not used in the calculation of formation constants, the hydrogen ion concentration was not required at any stage of the FICS calculations. However, the use of the sample diluent, or solvent, described in the method section, provides a convenient estimate of the hydrogen ion activity coefficient, γ_{H^+}.

The activity coefficient γ_{H^+} in a solution of 0.3 moles/kg $NaClO_4$ was estimated using the measured a_{H^+} based on NBS reference standards and the known concentration of nitric acid, determined by titration with NaOH. A solvent with an acid concentration of 1.16 x 10^{-4} moles/kg had a measured p_aH of 4.006, giving a γ_{H^+} of 0.85. A repeat of this procedure with a second solvent yielded an estimated γ_{H^+} of 0.91. Both of these values are higher than an expected γ_{H^+} of 0.7 in 0.3 moles/kg $NaClO_4$ (58).

The formation constants reported in this work may be converted to concentration constants by converting the hydrogen activity terms to hydrogen ion concentrations in the species that include protons. This step requires no modification in any parts of the FICS calculations.

CHAPTER IV

THE GRAVIMETRIC TITRATION SYSTEM

A. Introduction.

A titration system was designed for the specific purpose of collecting data suitable for the FICS evaluation procedure. Based on the discussions in Chapter II, such a system must be accurate and must be able to maintain one of the components in the sample at constant concentration. From a practical point of view, the system must also be fast and reliable. These requirements have been met by a titration system shown schematically in Figure 11. It consists of a digital pH meter, an electronic balance and the associated titrant delivery system, an auxiliary metal or ligand delivery system, a multiplexer, and a PDP-11/10 computer. The computer controlled the delivery of reagents from each of the titration units and, through the multiplexer, collected electrode potential and weight information from the pH meter and the electronic balance. This data was stored for future use with the FICS data handling method. The auxiliary delivery system, under computer instructions, maintained the metal or ligand concentration constant throughout the titration by compensating for dilution by titrant.

Two designs of gravimetric delivery units were built and tested. The first was based on a simple mechanical couple which opened and closed a pinch valve that controlled the titrant delivery, and the second employed an optical couple in which a beam of light triggered the opening and closing of the valve that controlled the flow of titrant. In both cases, the system was designed so that the weight of the delivered titrant was known.

Each part of the automatic gravimetric titration system will be considered in detail and the results of its application to simple acid-base titrations discussed in terms of the accuracy, precision, speed and reliability of the system.

B. Instrumentation.

The pH Measurement

The pH of the sample solution was measured with a Corning glass electrode and a Ag/AgCl reference electrode. The potential between these electrodes was measured by a Fisher 520 digital pH meter and the value of that potential, in millivolts (mV), was fed as a parallel binary coded decimal (BCD) signal to the multiplexer. This value was sampled by the PDP-11/10 under program control.

After each delivery of titrant a series of electrode potential measurements were taken and the average, the standard deviation and the linear regression of the points calculated. If the standard deviation and the drift in electrode potential were within the limits defined as equilibrium, the average of the set of measurements was taken as the electrode potential for that data point. This potential was converted to pH on the basis of electrode standardization with NBS buffers.

A Titrant Delivery System Based on a Mechanical Couple

A schematic diagram of the mechanical couple delivery system is shown in Figure 12. The titrant was delivered by gravity from a reservoir located on the balance pan of a Sartorius 3015 electronic balance, through a normally closed pinch valve, and to the sample cell.

The system consisted of four main components. The first was a polyethylene reservoir having an opening in the bottom for delivery of titrant and an opening at the top leading to a CO_2 trap. The titrant was delivered to the sample cell through Teflon tubing.

The second component of the system was a small, normally closed pinch valve. The mechanical energy necessary to open it was supplied by a motor driven device not actually sitting on the balance pan. With this design, a mechanical couple existed to the balance pan during the process of titrant delivery but not during the weighing. The mechanical couple provided the means for simultaneously transferring the information to start and stop the titrant

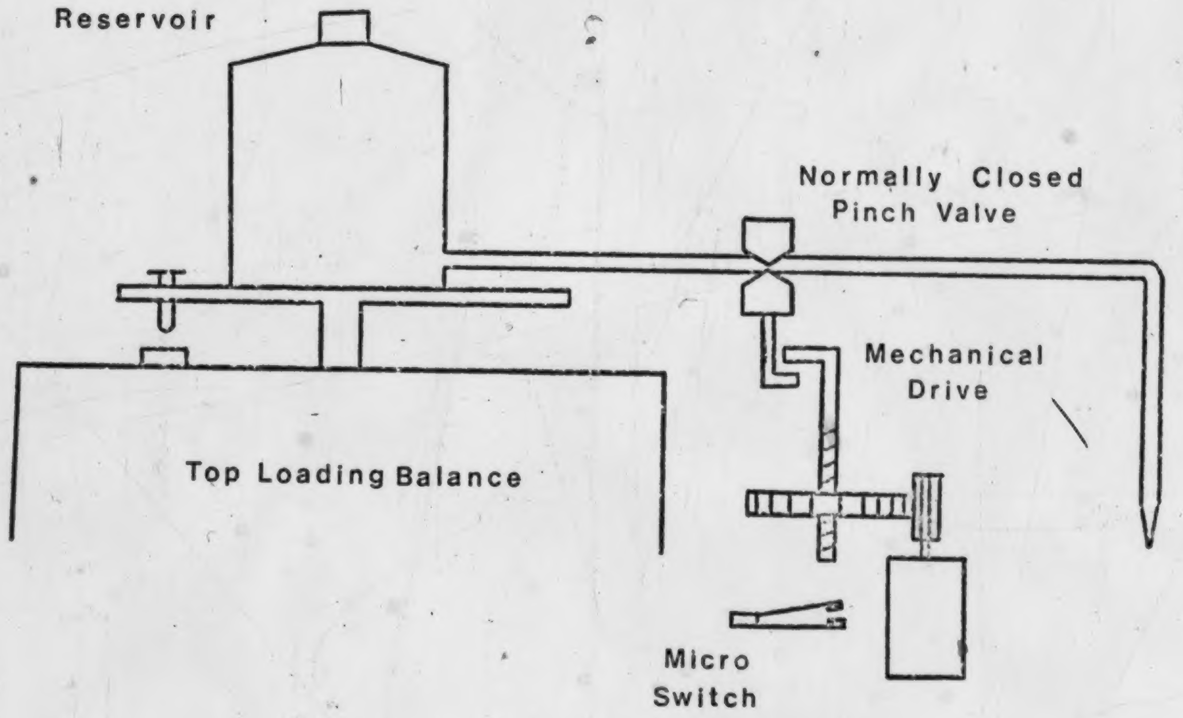

Reservoir
Normally Closed
Pinch Valve
Mechanical
Drive
Top Loading Balance
Micro
Switch

Figure 12. A Schematic Diagram of the Automatic Gravimetric Buret Based on a Mechanical Couple.

flow, and for transferring the mechanical energy necessary to open the valve. In the next section a device based on an optical couple will be discussed in which the information, but not the energy necessary to operate the valve was sent to the unit on the balance pan.

The third major component of the mechanical couple system was the motorized drive. This consisted of a small 1.5 V motor which drove a gear mechanism. The device was designed so that an arm pulled down to initiate titrant delivery and returned up when the motor was reversed. In the up position no contact was made with any part actually on the balance pan. When the motor pulled the arm down contact was made with the pinch valve, opening it and allowing the titrant to flow.

The power to the motor was supplied under program control. A simple circuit involving two relays and a battery allowed two bits of digital information to drive the motor in either direction or to stop it. The binary code 0,0 and 1,1 supplied no voltage to the motor, while 0,1 ran the motor in one direction and 1,0 ran it in the other. Delivery of titrant occurred through the following series of events : (a) 0,1 began the opening of the valve, (b) once the valve was open a microswitch signalled that the motor should be stopped, and the bits set to 0,0, (c) there was a delay while the titrant was delivered, (d) 1,0 for a period of time allowed the valve to close, and (e) 0,0 stopped the motor. At this point the routines designed to sample electrode potentials and balance reading were set into operation.

The time necessary for a delivery of titrant included delays at steps (a) and (d) during which the motor opened and closed the valve and a delay in step (c) while the titrant flowed. Steps (a) and (d) required 4.5 seconds each, and the delay in step (c) varied from several milliseconds to 20 seconds. The overall delivery time was a minimum of 9 seconds. The total time for each delivery in an actual titration also included titrant flow time, about 5 seconds for the balance pan to come to rest and a time for the electrodes to reach equilibrium. The total time between titrant additions then came to about 30 seconds.

The final major component of the system was a housing built around the titrator so that air currents would not affect the balance reading. Two housings were tested. The first covered all of the parts on the balance pan and, in addition, had a tube leading to the sample cell and coupling with it. This housing made the atmosphere over the sample cell continuous with that over the balance pan and nitrogen could be pumped over both simultaneously. The second housing, and the preferred one, also covered the balance pan but did not lead to the sample cell. A hole was provided for the titrant delivery tube, but the atmosphere over the balance was completely independent from that over the sample cell. Several problems were encountered with the first design. The balance was very sensitive to changes in humidity and temperature caused by the flowing nitrogen and, in addition, some buoyancy effects caused by the change in atmospheric components were observed.

The procedure used for the collection of data from the gravimetric delivery system was very similar to that for the pH meter. The BCD value of the weight of the unit on the balance pan was fed to the multiplexer. This value was sampled under computer control and the average of the series of weights taken as the best measure of the balance reading. Unlike the treatment of values from the pH meter, a standard deviation and slope were not taken. It was therefore necessary to delay the sampling a sufficient period of time to allow the balance pan to come to rest.

A Titrant Delivery System Based on an Optical Couple

The optical couple gravimetric delivery system is shown schematically in Figure 13. The titrant was delivered from a spring loaded syringe, through a solenoid valve, and to the titration cell.

The optical couple system consisted of four major components. The first was the titrant reservoir and delivery system. This consisted of a 10 ml Hamilton Gas-Tight syringe with a plunger modified to make it lighter, and Teflon tubing connected to the syringe by a tight Luer-type fitting and to the solenoid valve by regular high pressure liquid chromatography fittings. The tubing

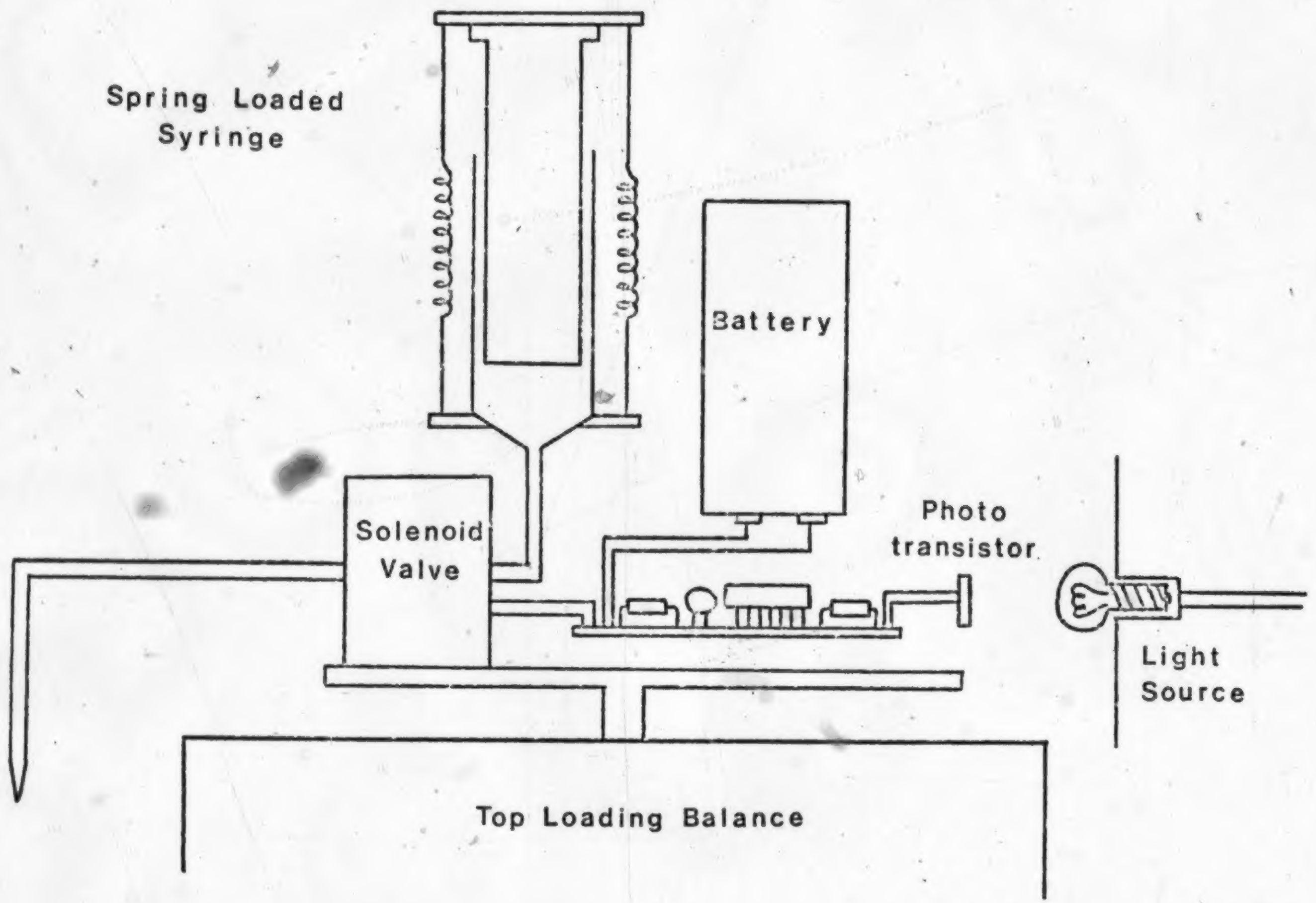

Spring Loaded
Syringe
Battery
Solenoid
Valve
Photo
transistor
Light
Source
Top Loading Balance

Figure 13. A Schematic Diagram of the Automatic Gravimetric Buret Based on an Optical Couple.

leading into the titration cell was drawn to a fine tip so that titrant was delivered as a tiny jet of liquid. This eliminated the problem of evaporation from a hanging drop, which may be serious with volatile solvents.

The second major component of the system was the valve and its power supply. A General Valve Corporation 2 way, 12 V DC, Iso-latch miniature solenoid valve was used to control the solution flow. The valve was opened by a single 12 V pulse and closed by another 12 V pulse. Current drain during each was about 700 ma, lasting approximately 150 msec. The power supply was a series of ten Eveready B225T, 1.25 V NiCd batteries. These batteries formed most of the weight of the device sitting on the balance pan.

The third major component of the system was the information transfer unit, in this case an optical couple. A single bit of digital information was used to control a light. Thus a 0 signalled the light to turn on and a 1 turned it off. A phototransistor located on the balance acted as a switch and controlled power to the solenoid valve. Therefore, the instructions to open and close the valve were supplied by the optical couple and the mechanical energy was supplied from the battery. This system had two major advantages; first, there was no mechanical interaction with the balance pan, making possible the simultaneous delivery of titrant and weight measurement and, second, the system was fast. The mechanical couple system required 4.5 seconds for each of the valve opening and valve closing steps, while each pulse to the optical couple required 150 msec. In addition, the mechanical couple system required approximately 5 seconds for the balance pan to come to rest, and less than one second for the optical system. Each titrant addition,therefore in theory, required 13 seconds less for the optical than the mechanical couple system (apart from actual titrant flow time, etc.)

The final component of the optical coupled system was the housing over the balance. This minimized drift due to air currents, and provided a convenient location to hold the light source as close as possible to the phototransistor. As discussed earlier, the preferred housing did not couple directly to the titration cell. A stream of N_2 could be blown over the sample solution, but not directed into the balance.

The collection of data from the optically coupled titrator was identical to that described for the mechanical coupled titrator. The only difference was that a shorter delay time was needed between the end of delivery and the sampling of the electronic balance output.

Each of the titrator designs had certain advantages and disadvantages. The optical system was capable of delivering titrant within short periods of time, saving about 13 seconds per delivery compared to the mechanical system. In fact however, not all of this time saving can be realized during a titration. The time of mixing of the sample solution and the rate of equilibration of the electrodes must also be taken into consideration. The rate at which the two systems are capable of operating is compared in more detail in the section dealing with the application of these units to simple acid-base titrations.

Another important difference between the titrators was the quantity of reagent held by each. The solenoid valve and the battery of the optical couple system were heavy, and as a result only 10 gm of titrant could be held in the syringe, suitable for one, or at the most two titrations (5-10 gm each). The mechanical system was simple and light, and held over 100 gm of titrant, enough to complete over ten titrations without refilling the reservoir.

The quantity of titrant delivered by both of these systems was controlled by the length of time the valve was held open, measured in 0.01 second intervals. The number of such intervals, or the time which the valve was held open, was manipulated from data point to data point according to the effect of the previous addition of titrant on the electrode potential. If the potential changed by less than a"minimum"value, then the next delivery would be twice as long. If the change was greater than a"maximum"limit, the delivery was halved, and if the change was greater than a "dangerous"level,then the delivery time was cut by five. With appropriately chosen levels, the systems could reliably handle even the abrupt end point of a titration of 4 moles/kg HNO_3 with 3 moles/kg NaOH. The systems did not once fail to get sufficient data to graphically determine the equivalence point.

The Multiplexer

A computer-experiment interface is designed to coordinate the transfer of digital information from one device to another. For example, a short dialogue might occur between the computer and the device supplying experimental data; first, the computer requests the data; second, the device prepares the information, perhaps doing an A/D conversion; third, the device sends a data ready signal; fourth, the computer reads in the data; and finally, the computer sends out a data accept signal. The role which the interface may play in this dialogue is the translation of the signals from one device into signals compatible with the next. This often means delaying of pulses, and changing of voltage levels or polarities.

The multiplexer used in the titration system served as the interface between the digital outputs of the balance and pH meter and the input of the LPS unit of the PDP-11. The circuitry of the multiplexer will be considered first and then the manner in which the sampling is done under program control.

The multiplexer made possible the transfer of data from each of several devices into a single input of the computer. The device employed for the titration system used 16 parallel 4 to 1 multiplexers, one for each bit of a 4 digit BCD number. Bit 00 from each of 4 different devices was fed to the first multiplexer, bit 01 from each into the next and so on. Two bits of information from the digital output of the LPS was used to select the device whose output was to be sampled. For example, a 0,0 signalled bit 00 from device 1 to appear on the output of the first multiplexer, bit 01 from device 1 to appear on the next output and so on. The outputs of the 16 channels were equivalent to the output of device 1. If the computer signalled 0,1, then the outputs of the 16 channels equalled the output of the second device, and so on. This system allowed sequential sampling of four different sets of BCD data. In this way it was possible to sample the output of the pH meter and the electronic balance. Due to the number of digits produced by the balance, its output was treated as the equivalent to two devices. The final channel was filled with zeros for debugging purposes.

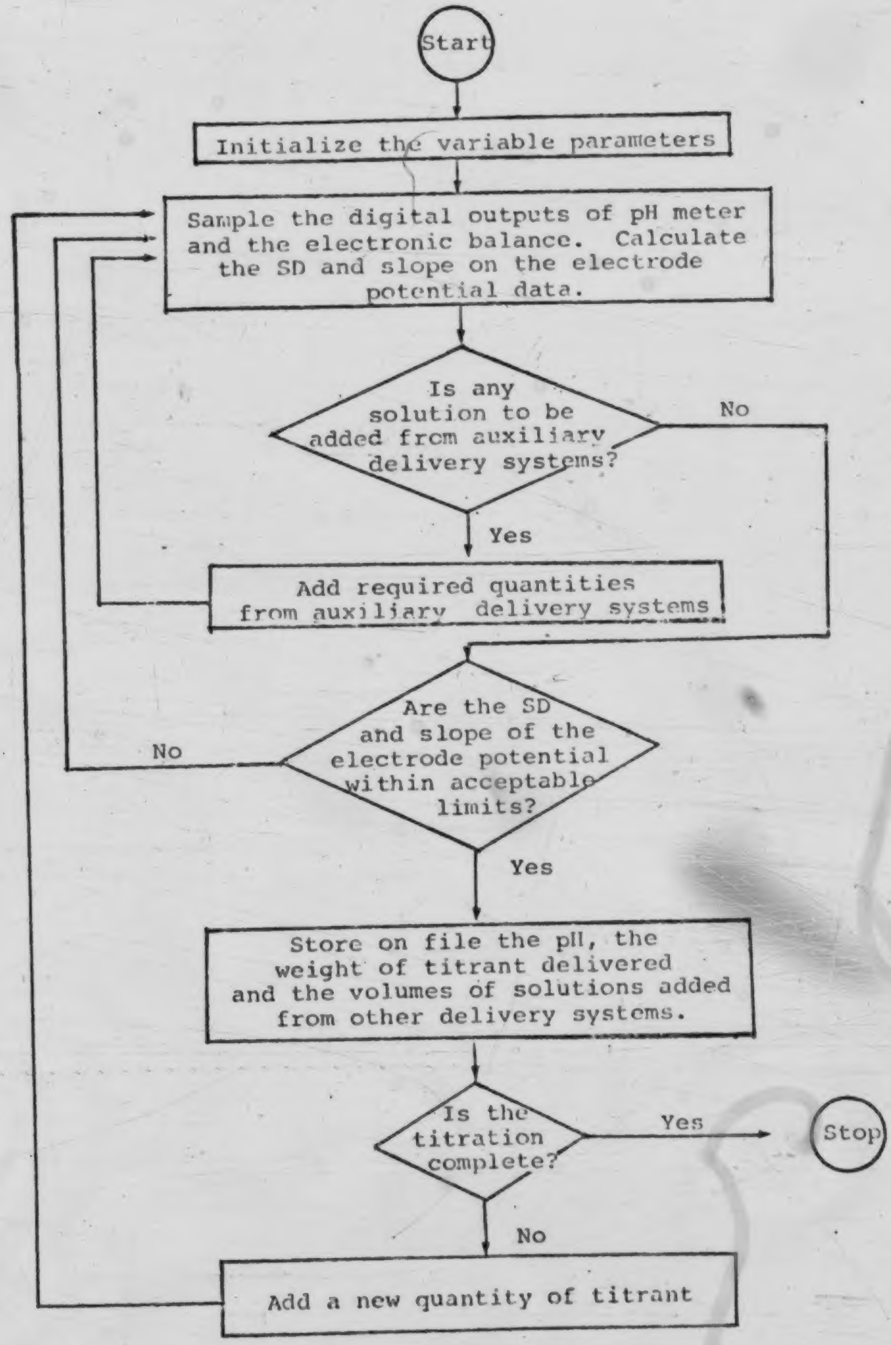
The digital input of the LPS unit was designed for two different modes of input. The first was the bit stimulus mode, in which the only bits recognized by the computer were those which made the 0 to 1 transition during the sampled period. This could be accomplished with the multiplexer by feeding the channel with all zeros to the LPS, then switching to the channels with the pH meter or balance. However, this turned out to be unreliable. To avoid this problem, the word stimulus mode was used, allowing the entire BCD code to be read directly. Unfortunately, the hardware of the LPS was not compatible with the aquisition routines of the language BASIC. For this reason the program running the titration, called TTR1 (see Appendix D), was written in FORTRAN. Because of its great flexibility, especially in the debugging and editing of programs, all other programs were written in BASIC. The data collected in FORTRAN was converted to a form compatible with BASIC with a short BASIC program called FCOPY (see Appendix E).

C. Software Control of the Automatic Weight Titrator System.

The titrator system operated entirely under the control of a single FORTRAN program called TTR1. While this program was being executed, the following operations would occur: (a) sampling of electrode potential and electronic balance output through the multiplexer, (b) anticipation of quantity of titrant and the delivery procedure, (c) calculation of quantities of solution to be added through auxiliary delivery systems (Mettler DV11) and subsequent delivery and, (d) the algebraic manipulation of the data (e.g. conversion of mV to pH) and the storage of that data on magnetic tape or floppy disc. Before considering the most important operations separately, an overview of the sequencing and timing of the entire program is essential. This is best described in flow chart form, Figure 14.

The initialization of variable parameters allowed the operator to fix the parameters at values suitable for the particular titration. These parameters included; (a) the

Figure 14. FLOW CHART OF PROGRAM TTR1


Start
Initialize the variable parameters
Sample the digital outputs of pH meter and the electronic balance. Calculate the SD and slope on the electrode potential data.
Is any solution to be added from auxiliary delivery systems?
No
Yes
Add required quantities from auxiliary delivery systems
Are the SD and slope of the electrode potential within acceptable limits?
No
Yes
Store on file the pH, the weight of titrant delivered and the volumes of solutions added from other delivery systems.
Is the titration complete?
Yes
Stop
No
Add a new quantity of titrant

slope and intercept determined by the calibration of the pH electrode (see program BUFFER, Appendix C) used to convert electrode potentials to pH, (b) the duration of the first delivery of titrant, (c) the "minimum," "maximum" and "dangerous" changes in the electrode potentials used to adjust the quantity of titrant delivered from point to point during the titration, (d) the number of readings of electrode potential to be taken in each sampling sequence, (e) the maximum allowed standard deviation and drift in electrode potential of the series sampled in (d), (f) the electrode potential which signalled the end of the titration, (g) the number of auxiliary burets to be operated (zero, one or two) and, (h) the concentrations of reagents in the auxiliary burets and the concentrations of the corresponding components in the sample cell, quantities which are needed in order to compensate for dilution.

With the values of these parameters fixed, TTR1 sampled the outputs of the pH meter and electronic balance and added the first titrant. The sampling was repeated, and the weight of delivered titrant calculated. If it was required by the FICS method, the quantity of reagent needed to compensate for dilution caused by this delivery of titrant was calculated and the necessary reagent added through the auxiliary delivery system. After this delivery, the sampling procedure was again repeated to determine the electrode potential. The standard deviation and drift in the electrode potential was calculated and, if the solution was not at equilibrium as defined by the operator, the sampling process was repeated. In this way the system collected equilibrium data after known additions of titrant and other reagents. Moreover, the conditions defined as equilibrium were identical from point to point, something not possible if the titration was run manually.

If the electrodes had reached equilibrium, TTR1 calculated the amount of titrant to be added in the next delivery, based on the effects of the previous addition. If the electrode potential changed by less than a"minimum" value, usually 4 or 5 mV, then the quantity of titrant was doubled; if the electrode potential had changed more than a "maximum"value,8 or 9 mV, then the quantity of titrant was cut in half; and if the change was over a"dangerous"limit, about 12 mV, the quantity of titrant was cut by five. The delivery was then made by sending the appropriate signals to the gravimetric delivery system. After the delivery the weight and electrode potential measurements were carried out once again. This entire process continued until the electrode potential passed the limit defined to be the end of the titration.

The remainder of this discussion will deal in detail only with those operations unique to the present system. That part of the code which is familiar to, or understood by the FORTRAN programmer will be omitted.

The program TTR1 communicated with external devices through the LPSDR option (sixteen bit buffered digital I/O) of the Laboratory Peripheral System. All of the program timing required the programmable real time clock, part of the LPSKW option. The version of TTR1 which ran the mechanical couple titrator also required a Schmitt trigger, a part of the LPSKW option.

Subroutine DELAY(TIME)

Those parts of TTR1 requiring delays called on subroutine DELAY(TIME), where TIME was an integer number of 0.01 second intervals. A delivery of 10 seconds then required a call of DELAY(1000). The FORTRAN statements were:

```
      DO 10 I=1, TIME
      ICMF = 0
      CALL SETR (5,0,1.,ICMF)
      CALL LWAIT (ICMF,0)
      CALL SETR(-1,,,)
10    CONTINUE
      RETURN
```

The first SETR statement set the programmable real time clock running at 100 Hz. The clock overflowed after 0.01 seconds and set ICMF equal to 1. The LWAIT statement made the computer wait until the value of ICMF no longer equalled 0. The second SETR statement stopped the clock. The DO loop then repeated this process for TIME intervals of 0.01 seconds.

Subroutine COLLECT(A,B)

Subroutine COLLECT(A,B) sampled the digital outputs of the pH meter and the electronic balance. It returned the electrode potential in A and weight reading in B. Since the output of the electronic balance was six BCD digits, two separate samplings were necessary, with algebraic combination of those to give a single value for B.

```
CALL IDOR (0,0,"3,"0)
CALL DELAY(1)
C = IDIR (0,0,-1,0)
CALL IDOR (0,0,"3,"1)
CALL DELAY (1)
B = IDIR (0,0,-1,0)
B = (10000*B)+C
CALL IDOR (0,0,"3,"2)
CALL DELAY (1)
A = IDIR (0,0,-1,0)
RETURN
```

The first IDOR statement set the final 2 bits of the digital output register to 0,0. These bits were the channel control of the multiplexer. The channel signalled by 0,0 was the last four digits of the electronic balance, which were read in by the IDIR statement and stored as the variable C. This process was repeated for the first digits from the electronic balance, stored as variable B, and for the output of the pH meter, stored as A. Values of B and C were combined to a single value in B, including all the six digits from the balance. The statements placing the decimal points in the correct place,and so on, have been omitted for simplicity. The DELAY statements allowed the multiplexer sufficient time to change channels.

Subroutine DELIVER(I)

The delivery of titrant from the weight titrator was controlled by DELIVER(I). The duration of the delivery, in 0.01 second units, was specified by the variable I. Delivery using the mechanical couple or the optical couple titrators were almost identical and, for simplicity, the optical system will be considered first.

```
      CALL IDOR (0,0,"4,"4)
      CALL DELAY (15)
      CALL IDOR (0,0,"4,"0)
      CALL DELAY (I)
      CALL IDOR (0,0,"4,"4)
      CALL DELAY (15)
      CALL IDOR (0,0,"4,"0)
      RETURN
```

The IDOR statements controlled a single bit of the digital output register. The first IDOR set the bit which turned on the light to open the valve. The light remained on for 150 msec and was turned off. A delay of I times 0.01 seconds occurred while the titrant flowed. A second flash of light of 150 msec duration closed the valve.

The mechanical couple system required that the IDOR statements control two bits. The first IDOR set the motor moving down, the second stopped it, the third set the motor in reverse and the final stopped it. The first DELAY was designed not to wait for the clock overflow, but for a signal from the Schmitt trigger. The second DELAY was exactly as above and the third was fixed at 450 rather than 15 as above.

Subroutine ADELIVER(N) and BDELIVER(N)

These routines were designed to deliver a series of pulses to the Mettler DV11 automatic piston buret. The FORTRAN code was:

```
      DO 10 I = 1, N
      CALL IDOR (0,0,"20,"20)
      CALL DELAY (5)
      CALL IDOR (0,0,"20,"0)
      CALL DELAY (5)
   10 CONTINUE
      RETURN
```

This DO loop produced a square wave with a period of 100 msec. The bit was repeatedly set and removed by the IDOR statements, with 50 msec delays between each. The subroutines differed only in the bit which they controlled. In this way, different volumes could be added from separate burets, as might be required for certain types of titration data.

D. Application of the Automatic Weight Titration Apparatus to Simple Acid-Base Determinations.

The reliability and the precision of the mechanical and the optical couple systems were evaluated using simple acid-base determinations. This work was designed to find those conditions, both from a chemical and from a software point of view, which would give the most valuable data. Since the immediate application of the system was the collection of data suitable for the FICS method, only discrete, equilibrium data point collection was studied. The apparatus has great potential for use in a variety of modes, including a mixture of continuous and discrete data point collection, pH stat type applications, fixed end point titrations and so on; however, these have not been considered. The apparatus is also readily adaptable to other methods of sample monitoring, for example spectroscopic, where digital information is accessible.

Those parameters which are of most importance in evaluating an automatic titration system include the rate of titration, the reliability of the entire system and, the accuracy and precision of the resulting data. Titrations of potassium hydrogen phthalate with sodium hydroxide and titrations of nitric acid solutions with sodium hydroxide were carried out to evaluate the performance of both designs of automatic weight titrators.

Results and Discussion

Consideration will first be given to the precision attained by the two basic designs of titrators, followed by a comparison of the different systems to illustrate their respective advantages and disadvantages.

Tables 23 and 24 are summaries of titrations of KHP and HNO_3 with NaOH using the mechanical couple system. The relative standard deviation of the KHP series was 0.06%, or about 4 mg of titrant (based on 7 gm total delivery). The relative standard deviation decreased to 0.04%, or about 1 mg for the strong acid-strong base titrations summarized in Table 24. Tables 25 and 26 are similar sets of data collected using the optical couple titrator. The standard deviations are essentially unchanged, about 4 mg of titrant for the KHP and about 2 mg of titrant for the HNO_3 titrations. Since the precision of the balance is 1 mg, this may be expected to be the limit of precision imposed on any set of titrations and this appears to be the case for strong acid-strong base titrations. The somewhat lower precision of the KHP titrations is due to a combination of factors including the lack of a sharp endpoint in the titration of KHP and the susceptibility of these titrations to errors introduced by impurities or CO_2 in the reagents since the equivalence

Table 23. Precision of the Standardization of Sodium Hydroxide with Potassium Hydrogen Phthalate Using the Weight Titrator Based on a Mechanical Couple.

Sample	Weight KHP, gm	Consumption of NaOH, gm	Calculated NaOH Concentration moles/kg
1	0.4994	6.657	0.3673
2	0.4996	6.656	0.3675
3	0.4856	6.464	0.3678
4	0.5056	6.733	0.3677
5	0.5891	7.850	0.3674
6	0.6113	8.137	0.3678
		Average	0.3676
		Relative Standard Deviation	0.06%

Table 24. Precision of the Strong Acid-Strong Base Titration Using the Mechanical Couple Weight Titrator.

Sample	Weight HNO_3, gm	Consumption of NaOH[a], gm	Calculated HNO_3 Concentration moles/kg
1	7.9476	3.508	1.3242
2	7.6372	3.371	1.3241
3	5.2433	2.314	1.3240
4	5.4243	2.392	1.3229
5	7.1055	3.135	1.3236
6	7.1929	3.172	1.3230
7	6.6570	2.937	1.3236
8	7.5852	3.347	1.3237
		Average	1.3236
		Relative Standard Deviation	0.04%

[a] Concentration of NaOH was 3.0 moles/kg.

Table 25. Precision of the Standardization of Sodium Hydroxide with Potassium Hydrogen Phthalate Using the Weight Titrator Based on an Optical Couple.

Sample	Weight KHP, gm	Consumption of NaOH, gm	Calculated NaOH Concentration moles/kg
1	0.5159	6.427	0.3930
2	0.5737	7.150	0.3929
3	0.6801	8.482	0.3926
4	0.6739	8.405	0.3926
5	0.5179	6.452	0.3930
6	0.6720	8.378	0.3927
7	0.4927	6.140	0.3929
8	0.4990	6.214	0.3932
9	0.5074	6.319	0.3932
		Average	0.3929
		Relative Standard Deviation	0.06%

Table 26. Precision of the Strong Acid-Strong Base Titration Using the Optical Couple Weight Titrator.

Sample	Weight HNO_3, gm	Consumption of NaOH[a], gm	Calculated HNO_3 Concentration moles/kg
1	6.7709	9.962	4.4139
2	6.6627	9.806	4.4153
3	7.1958	10.589	4.4146
4	6.7530	9.938	4.4149
5	7.0166	10.328	4.4158
6	6.8383	10.064	4.4151
7	6.8340	10.057	4.4148
8	7.0527	10.380	4.4153
9	7.1325	10.501	4.4168
		Average	4.4152
		Relative Standard Deviation	0.018%

[a] Concentration of NaOH was 3.0 moles/kg.

point is above pH 7.0. Error may also have been introduced in the sample preparation stage requiring the weighing of small quantities of solid KHP.

The titrators were equivalent from the point of view of accuracy and precision, both devices being limited by the same factors, namely either the chemistry of the system, or by the sensitivity of the balance. The mechanical couple system required a minimum of 9 seconds to deliver titrant, plus 5 seconds for the balance pan to come to rest and 6 seconds to sample the electrode potential. This resulted in a basic delivery time of 20 seconds. The optical couple required only 7 seconds for the same operations, saving 13 seconds. These were not the only procedures which required considerable time however. The titrant delivery consumed a large amount of time. In order that a minimum delivery of 5 mg could be attained, the same flow rate delivered 0.5 gm in 30 seconds. Both the optical and mechanical couple devices were limited to delivery at that rate. A significant amount of time was also required for the electrodes to reach equilibrium near the equivalence point. This was in part due to the rate of mixing of reagents and could be improved with better cell design.

The delay during which the electrodes came to equilibrium removed much of the speed advantage of the optical couple titrator. After the addition of titrant both systems required several seconds of delay before sampling the electrode potential; however, because of its slowness the mechanical couple titrator needed a slightly shorter delay. If the delay time used with the optical system was shortened, the electrodes would not be at equilibrium and the sampling step would have to be repeated, resulting in a longer effective delivery time than that of the mechanical couple. Since about 60 data points were collected for the FICS method, a few seconds per delivery could considerably change the total time required for the titration.

The total time for titration using the optical couple ranged from 10 minutes, collecting 30 data points suitable for equivalence point measurement, to about 40 minutes to collect 50 data points suitable for use with the FICS method which also included delivery from one extra buret. The mechanical couple system required from 20 minutes to 60 minutes for the same experiments. The optical couple system required about 20 minutes to run each of the titrations shown in Tables 25 and 26 (35 - 40 data points).

In addition to the actual running time of the titrator, time was consumed in manual operations. This included removing the titrated sample, rinsing the electrodes and reassembling the apparatus with a new sample. The optical couple titrator also required that the syringe be refilled between experiments. Futher refinements of this system should allow, as did the mechanical couple titrator, multiple titrations with a single reservoir of reagent. This is especially true if the apparatus is to be applied to routine gravimetric reagent handling. The total time necessary to perform the nine titrations in Table 26 was 3.5 hours. This is slow compared to manual, colourimetric end point titrations with a buret, about equal in time for the same titrations using an automatic piston buret, but much more rapid than the equivalent manual gravimetric titrations. This increase in rate over manual gravimetric titrations was attained with no sacrifice in precision.

Both titrators were found to be very reliable, both from the point of view of mechanical and electrical operation and also in the ability of the entire unit to respond to very sharp equivalence points. For example in the titrations shown in Table 26 approximately 35 data points were collected, yet 12 of those were within ±0.4 gm of the end point (total titrant delivery, 10 gm). This allowed graphical determination of the end point using a significant number of the total data points. The system was able to respond to the approach of the sharp change of potential without either extreme caution, that is, without giving an excessively large number of points, or uncontrollably large additions which would make graphical determination of the end point impossible. The system never failed to collect sufficient data to allow graphical end point determination.

Several potential problems and limitations of the system were encountered. These were involved primarily with the long term behavior of the electronic balance. It was found that a very considerable rate of drift could be induced by a change in humidity. An increase in humidity brought about an apparent decrease in the weight reading of as much as 5 mg in 15 minutes, while a decrease in humidity brought an apparent increase in weight. The effect of temperature was not specifically studied but it is certainly expected to have similar effects.

These problems introduced difficulty in the design of a controlled atmosphere titration cell. First, there must not be any mechanical connections between the delivery tube and the cell. This precludes a tight seal around the opening where the titrant is delivered to the cell. The alternatives are therefore to either pump a sufficient quantity of inert gas over the cell to minimize entry of atmospheric components into the cell or to contain the titrant delivery system and the cell in a common housing. The former alternative was taken after work with a common housing revealed that very elaborate means would have to be taken to control the atmosphere sufficiently closely to stop balance drift. In certain applications, for example with volatile or reactive substances the latter method may be employed if the titration is carried out sufficiently rapidly that drift error is negligible for the desired precision of results.

Summary

The nature of the FICS data handling procedure has placed several requirements on a titration system. The requirements for accuracy have been met by the design of two forms of automated gravimetric titrators and by the complete automation of data handling. The system was fast and reliable, characteristic of computer controlled experiments, and made decisions concerning equilibrium of the electrode potentials and quantities of material to be delivered by all the burets with a speed and reliability impossible manually.

The automated gravimetric titrator systems based on optical and mechanical couples were tested on simple acid-base titrations. From these experiments optimum values of delay intervals, criteria for equilibria, and other important parameters were evaluated. Several important instrumental problems including the design of the housing around the titrator were also resolved. The system based on the optical couple performed better than did the mechanical couple, especially in terms of speed, and was used in all later experiments.

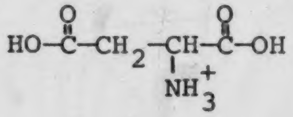
In the next chapter a study of the complexation of zinc with aspartic acid is described. The data was collected using the optical couple titrator system and the treatment of data was carried out using the FICS method described in Chapter II.

CHAPTER V

APPLICATION OF THE FICS METHOD TO THE DETERMINATION OF THE STABILITY CONSTANTS OF COMPLEXES BETWEEN ASPARTIC ACID AND ZINC

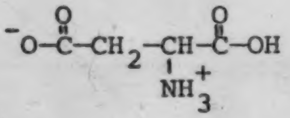
A. Introduction.

Aspartic acid is a potentially tridentate ligand with the structure shown below.

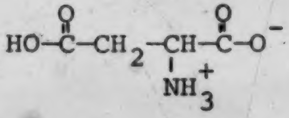


$$HO-\overset{\overset{O}{\|}}{C}-CH_2-\underset{\underset{NH_3^+}{|}}{CH}-\overset{\overset{O}{\|}}{C}-OH$$

Binding to metal ions may occur through the β carboxyl group, and the α carboxyl and α amino groups. At low pH all of the groups are protonated, and at pH values near 2,4 and 9, the α carboxyl, β carboxyl, and amino protons, respectively, are removed. To be more precise this acid dissociation scheme must be described in terms of a series of microscopic acid dissociations. For aspartic acid the first proton removed may be either the α or β carboxyl protons. The structures of these are shown below.

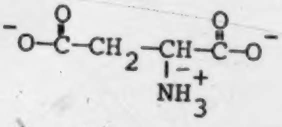


$$^{-}O-\overset{\overset{O}{\|}}{C}-CH_2-\underset{\underset{NH_3^+}{|}}{CH}-\overset{\overset{O}{\|}}{C}-OH$$



$$HO-\overset{\overset{O}{\|}}{C}-CH_2-\underset{\underset{NH_3^+}{|}}{CH}-\overset{\overset{O}{\|}}{C}-O^{-}$$

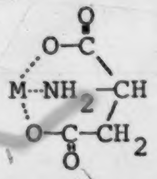
Both of these species have the stoichiometry H_2L. Removal of a second proton will lead to a single form of HL.



$${}^{-}O-\overset{O}{\overset{\|}{C}}-CH_2-\underset{NH_3^{+}}{\underset{|}{CH}}-\overset{O}{\overset{\|}{C}}-O^{-}$$

While there are three possible forms of HL, due to the low acidity of the amino group this form will by far predominate.

In a solution of metal ions, the metal ion may displace carboxyl or amino protons and bind to those groups. If all three protons have been displaced the molecule may easily bind to the metal through the three groups simultaneously. The structure of aspartic acid is such that it very neatly surrounds the metal on three adjacent sides.



Two ligands may bind simultaneously, completing the octahedral coordination sphere preferred by a number of metal ions. The two strongest complexes of aspartic acid with most metal ions are therefore ML and ML_2.

The zinc-aspartic acid system was chosen for the study and application of several of the procedures developed as part of the FICS method. A relatively simple chemical system was necessary in order to avoid, as much as possible, difficulty in assigning a model to the behavior of the system. The information this study was intended to provide included: (a) the accuracy of experimental results which could be obtained with the system described in Chapter IV, (b) the severity of curvature of the (dC_H/dC_L) and (dC_H/dC_M) functions at various ligand to metal ratios, (c) the applicability of the methods previously discussed with respect to the determination of $p[L]_o$ and $p[M]_o$, and (d) the reliability of the constants derived with the available data collection methods, and the experimental modifications necessary for the study of the chemistry of more complex systems.

B. Determination of the Acid Dissociation Constants of Aspartic Acid

A series of solutions containing from 2.643×10^{-3} to 5.908×10^{-3} moles/kg of aspartic acid were titrated with NaOH from pH 3.5 to 11.0. The FICS method can most easily be applied to the titration of an acidic ligand since: (a) no acid needs to be added to the solution to bring it to pH_o, making the solution composition data more accurate, (b) the concentration of the acid in each sample can be determined from the equivalence point following the consumption of the carboxyl proton, also avoiding the errors normally expected in the solution preparation, and (c) the range of C_L can be unlimited since (dC_H/dC_L) is linear. This final factor is of considerable importance since the precision of (dC_H/dC_L) or alternatively $\Delta C_H/\Delta C_L$

is very much dependent on the magnitudes of ΔC_H and ΔC_L. Both must have good accuracy before (dC_H/dC_L) is known with some reliability.

The raw titration data was handled in exactly the manner described in Chapter II, and involved the calculation of the total titratable proton concentration, C_H, at each data point and interpolation between those points to determine C_H at exact pH values. The raw data was collected at intervals of 0.1 to 0.2 pH units, and the C_H values required for evaluation of (dC_H/dC_L) were obtained by interpolation at exact 0.1 pH intervals. Figure 15 shows the variation in C_H with change in the total ligand concentration. As expected, the plots are linear and indicate that the experimental methods used can provide very accurate data since within the sensitivity of the graphical method, all of the data points fell along the lines. More exact mathematical treatment revealed that the fit of the points to a straight line was less precise at extremes of pH. This is to be expected because at extremes of pH large quantities of titrant are required to change the pH slightly, and as a result, small errors in pH produce increasingly large errors in C_H. This introduces uncertainty in the derivative (dC_H/dC_L) at pH values below 4 and above 10.

Figure 16 summarizes the derivatives (dC_H/dC_L) as a function of pH. With those values the concentration of free aspartic acid, [L], was calculated throughout the pH range of the available data. The $p[L]_o$ was first chosen assuming

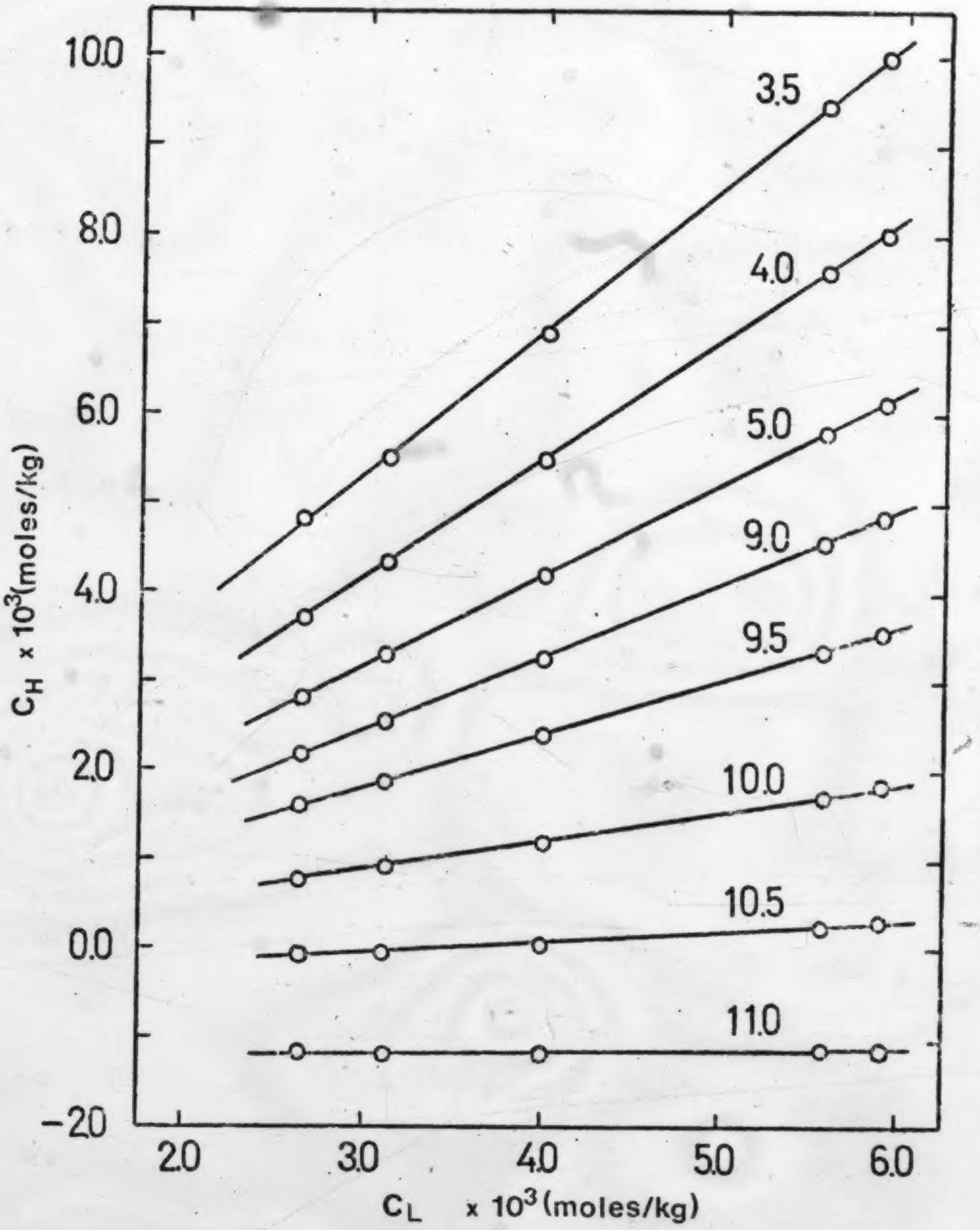


Figure 15. C_H vs C_L at various pH for Aspartic Acid. The values of the derivatives, (dC_H/dC_L), are shown in Figure 16.

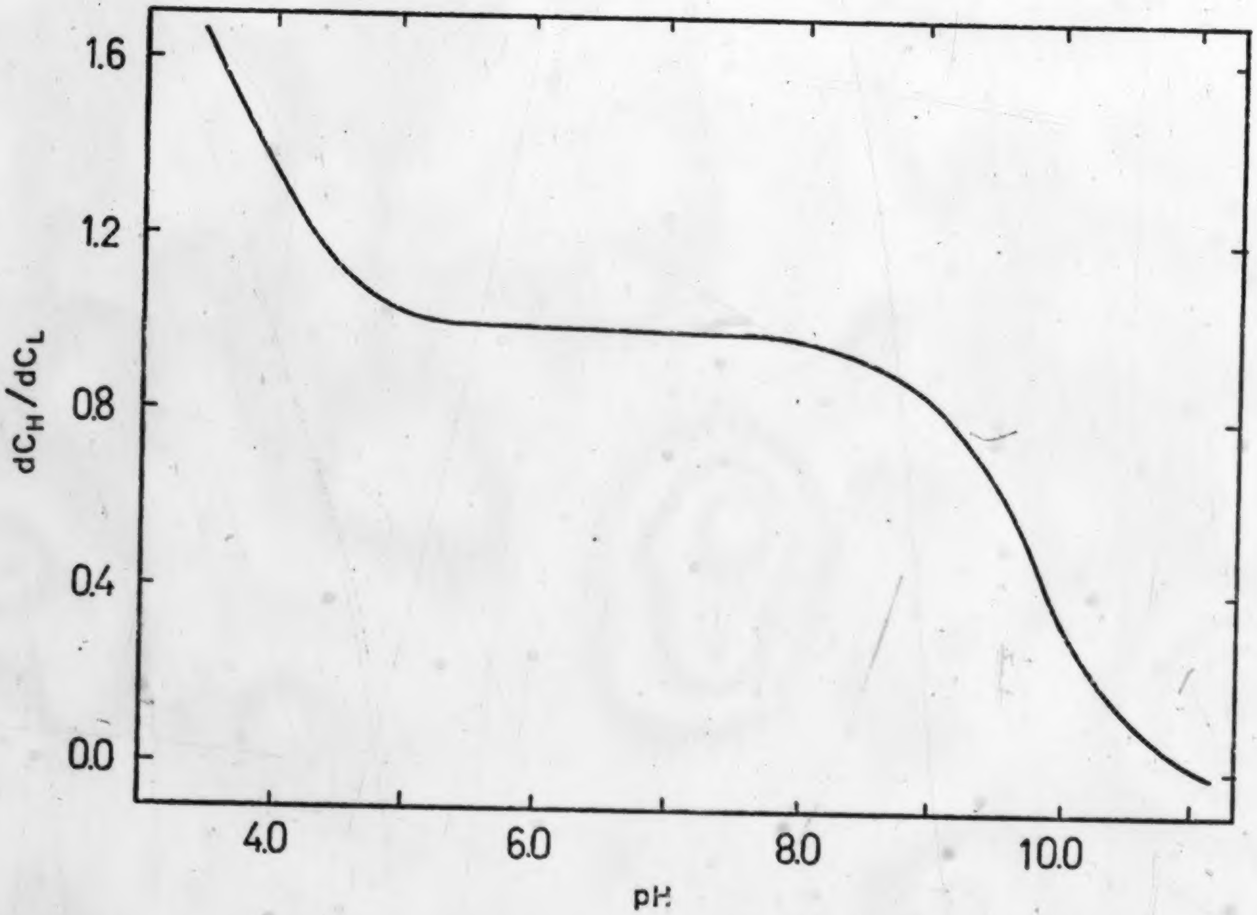


Figure 16. (dC_H/dC_L) as a function of pH for aspartic acid.

no protonation of the ligand at pH 11.0. The acid dissociation constants were calculated on the basis of that $p[L]_o$ and from the new set of constants a new $p[L]_o$ was calculated. This process required three stages, in which $p[L]_o$ was 3.000, 3.006 and 3.020, and the standard deviation of the fits varied as 3%, 2.3% and 1.8% respectively. Using a $p[L]_o$ of 3.020, a new $p[L]_o$ of 3.020 was calculated. This procedure worked as predicted by the studies using simulated titration curves. The resulting protonation constants are shown in Table 27.

C. The Formation of Complexes between Zinc and Aspartic Acid.

Several aspects of the FICS procedure discussed with respect to simulated titration curves were investigated using the zinc-aspartic acid system. It was of interest to determine the nature of the problems of curvature of the function of C_H with change in C_L for this metal-ligand system. A series of titrations was performed with the concentration of zinc fixed at 9.370×10^{-4} moles/kg and the concentration of ligand varying from 1.1×10^{-3} to 5.6×10^{-3} moles/kg. The concentration of total titratable proton, C_H, was calculated; the variation of C_H at several pH values is shown in Figure 17. There were no regions of sharp curvature, however, some curvature in the region of 2:1 ligand to metal ratio developed in the pH range above 7.0. This is expected because the complex ML_2 predominates

Table 27. Determination of the Protonation Constants of Aspartic Acid.

Species	log (β)		
	$p[L]_o = 3.000$	$p[L]_o = 3.006$	$p[L]_o = 3.020$
HL	9.652	9.663	9.678
H_2L	13.335	13.344	13.356
SD of fit:	3.0%	2.3%	1.8%

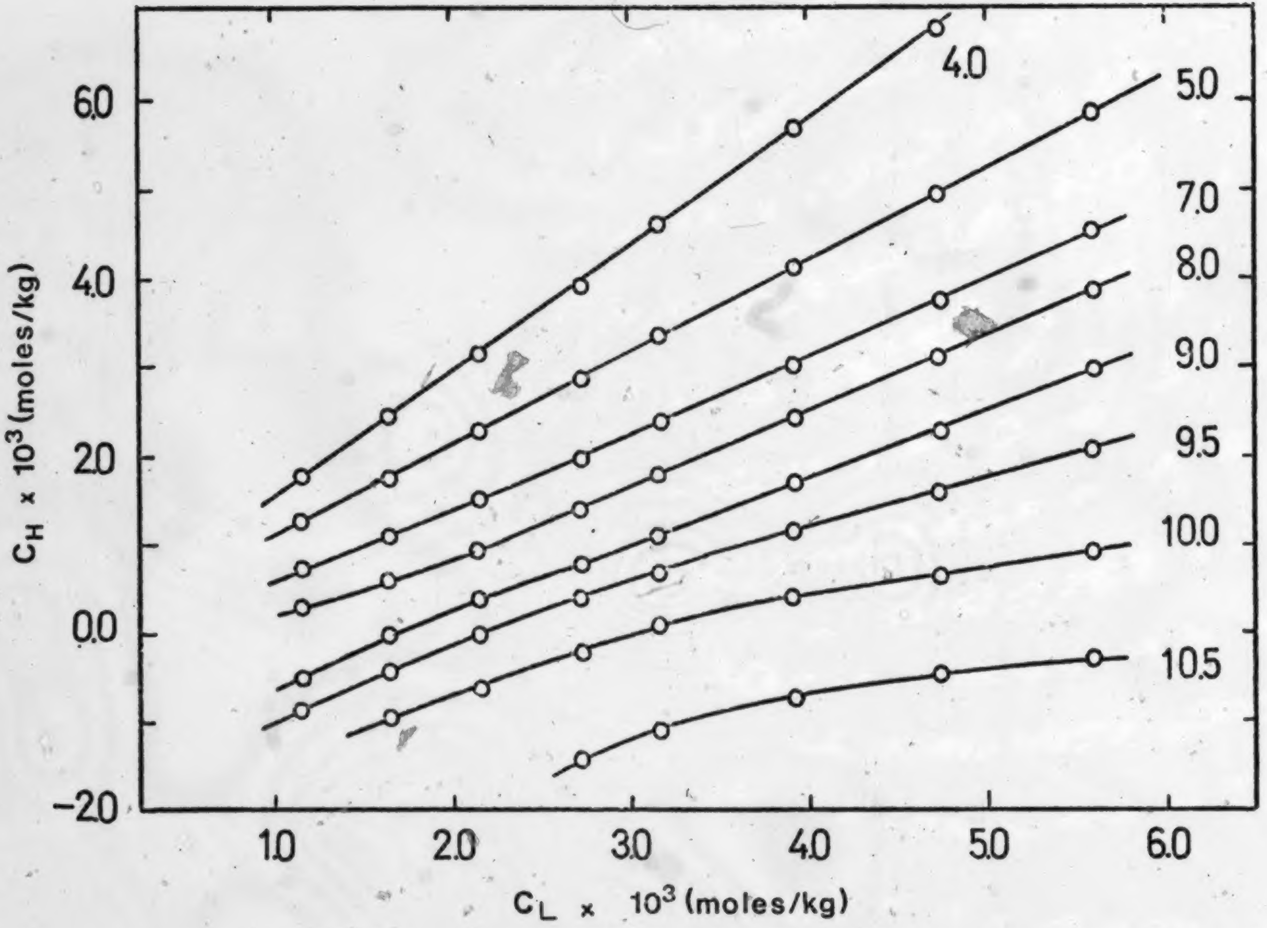


Figure 17. C_H vs C_L at several pH values for the Zinc-Aspartic Acid system. The Zinc concentration was held constant at 9.370×10^{-4} moles/kg.

in that pH range. At higher pH precipitation occurred in all of the titrations; the effects of this can be seen in Figure 17. The curve for pH 8.0 behaves as expected for a system containing the species ML and ML_2 (see Figure 1, pH 5.0). However, at pH 9.0 the effect of precipitation at low C_L tends to turn the C_H curve towards lower C_H. This curvature becomes more severe as the pH is increased, and at pH 10.5 the curve has a pronounced downward curvature. These results are reasonable if the precipitation of metal hydroxide is considered to be equivalent to the formation of soluble hydroxy complexes which, as discussed in Chapter II, contribute a negative value to the concentration of titratable protons. C_H therefore becomes more negative. In addition, it should be noted that the effect of precipitation on the C_H vs C_L curves begins at low C_L at pH 9.0 and progresses towards higher C_L as the pH is raised. This is because precipitation of the metal is inhibited by the increasing concentration of ligand. On the basis of Figure 17, the FICS method could most effectively be used above a 3:1 ligand to metal ratio and up to a pH of 9.5.

Ten titrations were performed; five at a constant zinc concentration of 9.370×10^{-4} moles/kg and five at a constant aspartic acid concentration of 5.308×10^{-3} moles/kg. Figures 18 and 19 are plots of C_H vs C_L and C_H vs C_M calculated from the raw titration data. The slopes of these lines were calculated by a linear least squares procedure. Figure 20 is the results of those calculations shown as a

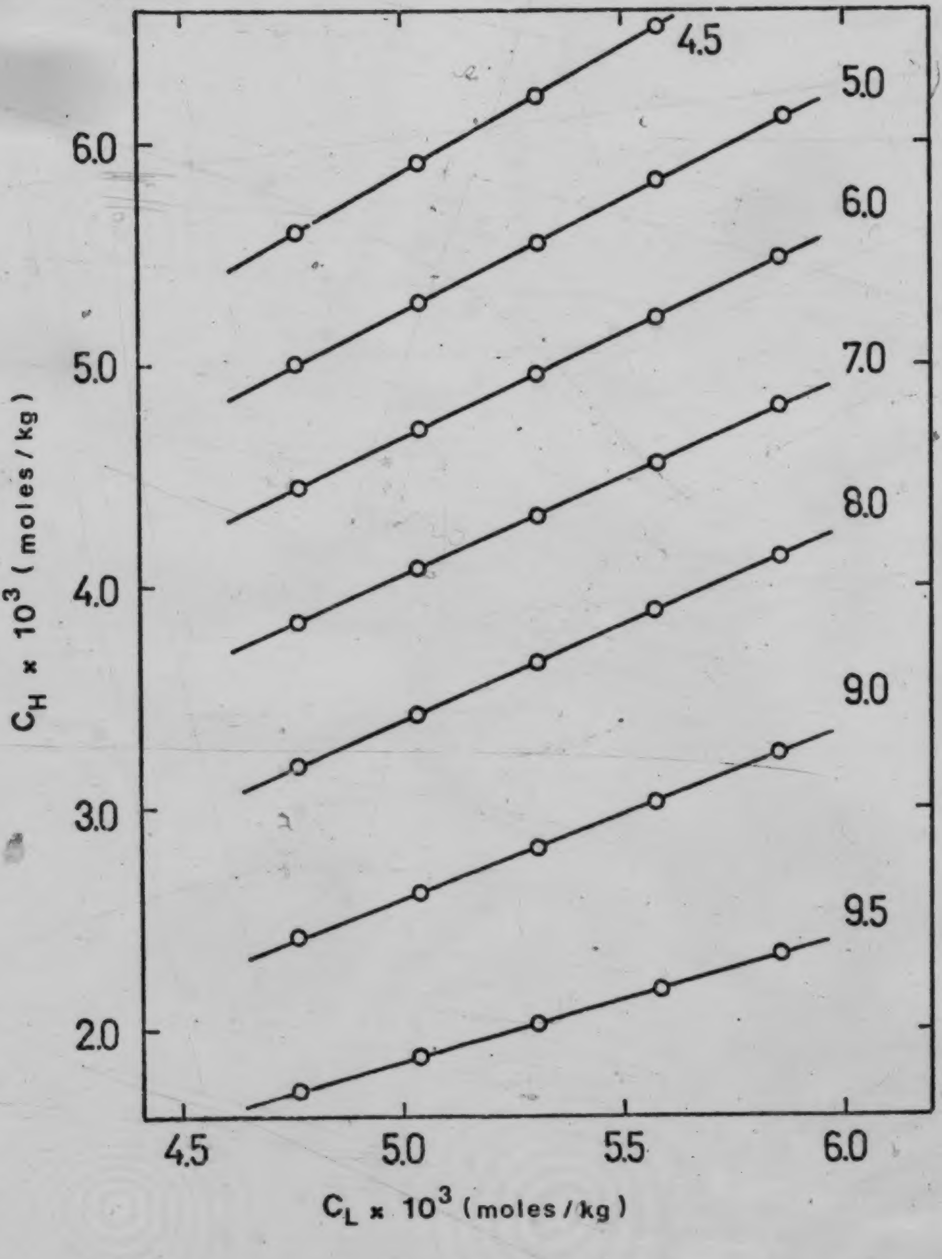


Figure 18. C_H as a function of the total concentration of aspartic acid (C_L) for the Zinc-Aspartic Acid system. The total zinc concentration was 9.370×10^{-4} moles/kg.

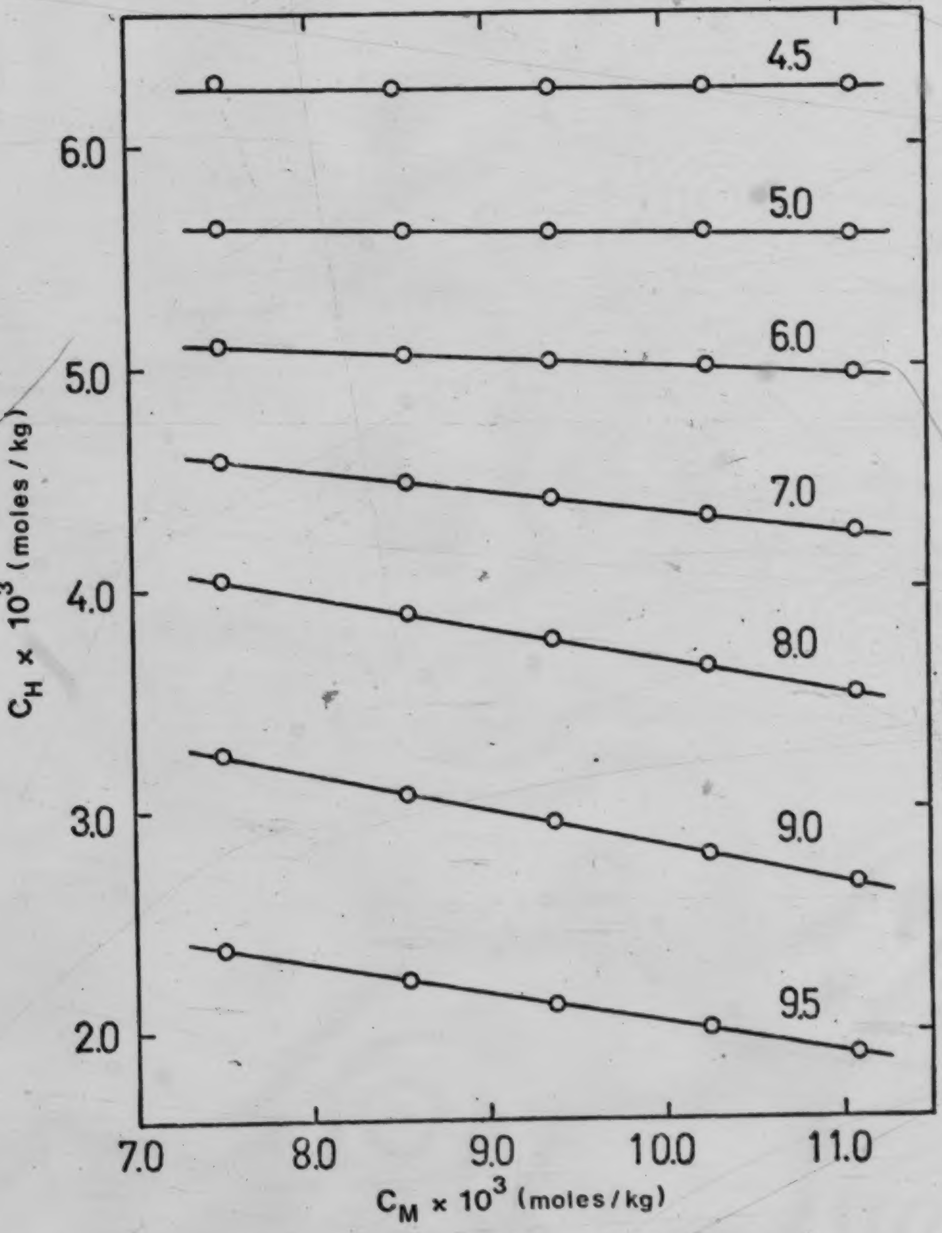


Figure 19. C_H as a function of the total concentration of zinc (C_M) for the Zinc-Aspartic Acid system. The total aspartic acid concentration was 5.308×10^{-3} moles/kg.

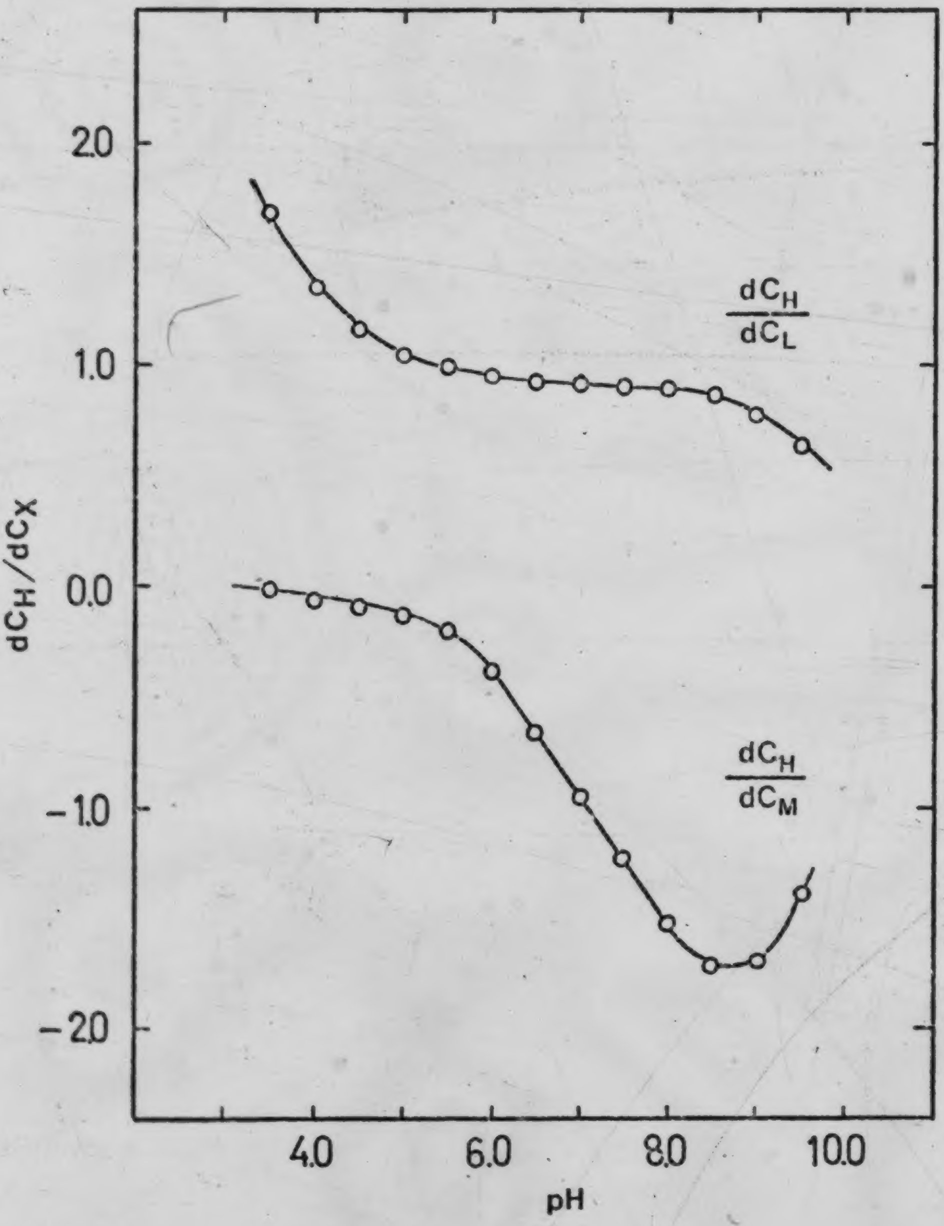


Figure 20. (dC_H/dC_L) and (dC_H/dC_M) as a function of pH for the Zinc-Aspartic Acid system. The derivatives were evaluated at a 5:1 ligand to metal ratio.

function of pH. The curve for (dC_H/dC_L) is very similar to that of aspartic acid alone, shown in Figure 16, because the titrations were carried out with a large excess of ligand; however, the curves are not identical. The curve for (dC_H/dC_M) indicates that slight complexation occurs in the range of pH 4.0 to 5.0 since (dC_H/dC_M) is not exactly zero, and that the strongest complexation begins at pH 5.0 where the derivatives become much different from zero. On the basis of the tabulated function (dC_H/dC_L) and (dC_H/dC_M), and $p[L]_o$ and $p[M]_o$ calculated assuming no complexation at pH_o, the free zinc and free aspartic acid concentrations were calculated using Equations 9 and 10. The [L] and [M] values were then used to aid in the selection of a model of complexation.

Aspartic acid has three coordinating groups, two carboxyl groups and one amino group. Near pH 5.0 the uncomplexed ligand contains a single proton on the amino group. This proton may be displaced by a metal ion to give the complex ML. Two of the tridentate aspartic acid molecules may easily surround a metal ion, forming the complex ML_2. These are the only complexes reported for zinc and aspartic acid. Other possible complexes, reported with other metals, and with glutamic acid, a ligand very similar to aspartic acid, include HML where the amino group remains protonated and ML_3 where presumably the β or γ carboxyl group is dislodged and the ligand is bidentate. The first step in deciding a model for the zinc-aspartic acid system was to attempt to calculate constants for all four complexes, HML, ML, ML_2 and ML_3, and to compare standard deviation of the fits to the total ligand and metal in solution. This process is summarized in Table 28. In the first attempt only the complexes ML and ML_2 and the protonation constants of the ligand were considered. The standard deviation using the ligand and metal mass balances from pH 4.0 to 8.0 were 0.15% and 7.22% respectively. Next, the species HML was added for the same pH range; the fits were 0.10% and 3.83% respectively, a considerable improvement. This same model was tried for the pH range 4.0 - 9.0 but the constants for both HML and ML were negative. Finally, the species ML_3 was included, using the pH range from 4.0 to 9.0. and the standard deviation of the fits were 0.22% and 3.52%. This indicated that all four complexes might be present in solution.

The delta quantities, ΔC_H, ΔC_L and ΔC_M described in Chapter II, were used to further study the model of complexation. Figure 21 shows the values of these quantities calculated on the basis of the [L] and [M] data discussed above and estimated values of the ligand protonation constants. Below pH 5.0 there appears to be only slight complexation, as predicted by the (dC_H/dC_M) plot in Figure 20. Between pH 5.0 and 7.0 ΔC_L and ΔC_M are about equal, indicating that the complex ML predominates in that region. The ratio of ΔC_L to ΔC_M increases from that point on to over 2:1 at pH 9.0. The complex ML_2 predominates in

Table 28. Determination of the Formation Constants of Complexes of Zinc and Aspartic Acid with pH_o 4.0

Species	log (β)		
	$\sum L$	$\sum M$	$\sum ML$
H_2L^a	13.38		13.37
HL	9.58		9.58
ML	5.80	5.84	5.80
ML_2	10.24	10.05	10.22
SD of fit:	0.15%	7.22%	SD_L=0.55% SD_M=9.66%
H_2L^b	13.37		13.36
HL	9.56		9.58
HML	11.36	10.96	10.89
ML	5.89	5.81	5.78
ML_2	10.26	10.10	10.24
SD of fit	0.10%	3.83%	SD_L=0.73% SD_M=7.28%
H_2L^c	13.37		13.35
HL	9.57		9.58
HML	11.19	10.97	10.81
ML	5.86	5.81	5.78
ML_2	10.25	10.11	10.21
ML_3	12.87	13.02	12.92
SD of fit	0.22%	3.52%	SD_L=1.00% SD_M=8.75%

Table 28 (continued)

[a] $p[L]_o = 8.068$, $p[M]_o = 3.028$ at $pH_0 = 4.0$
Range of data: pH 4.0 - 8.0

[b] as in a. Species HML has been added to the model of complexation.

[c] as in a and b, except that the pH range has been expanded to 4.0 - 9.0 and species ML_3 included in the model of complexation. The models from a and b were not suitable for this pH range.

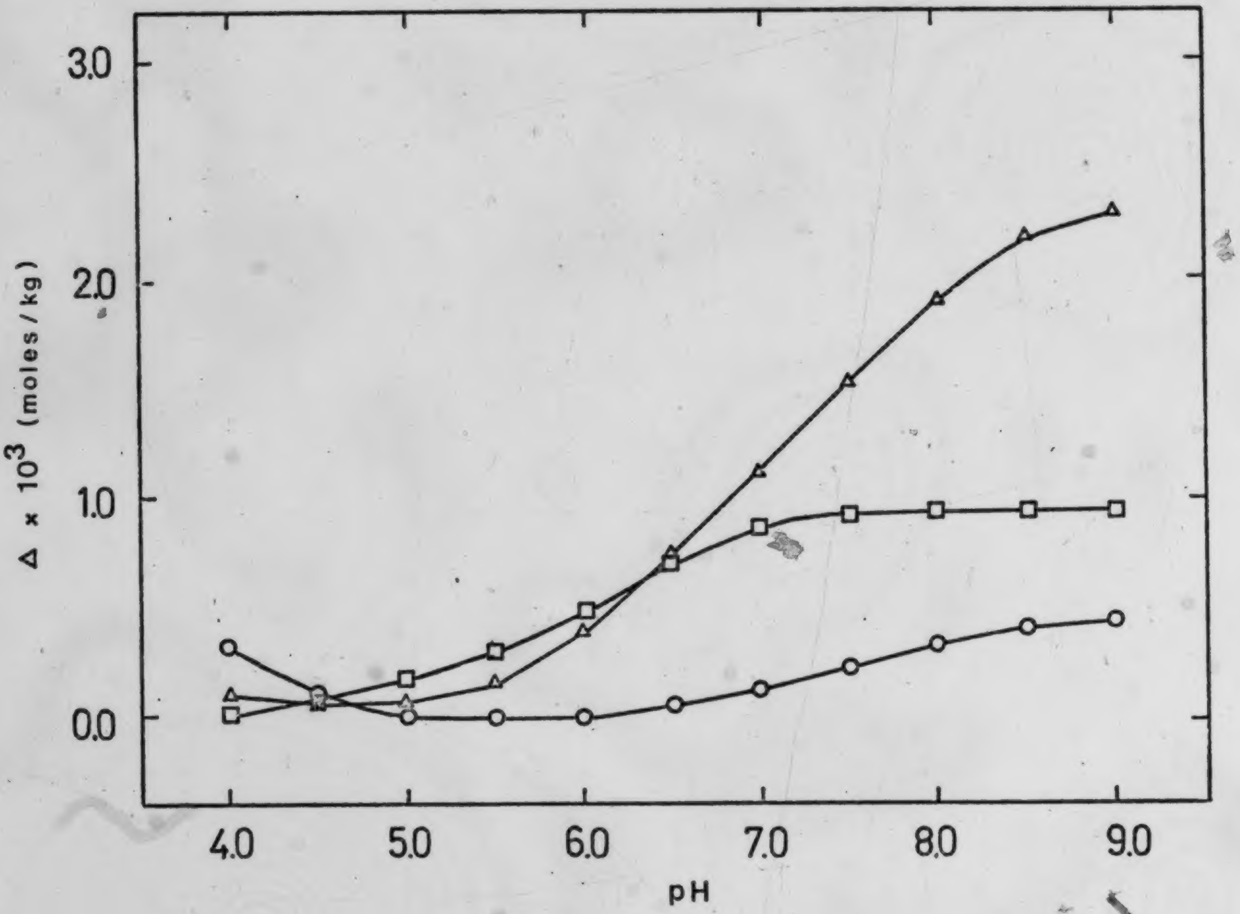


Figure 21. ΔC_H, ○; ΔC_L, ▲ ;and ΔC_M, ▫ ; for the Zinc-Aspartic Acid system. The delta quantities have been calculated assuming no complexes are forming.

this pH range and a complex with a higher ligand to metal ratio may be forming.

The ΔC_H curve decreases from pH 4.0 to 5.0, remains constant from 5.0 to 6.0 and gradually increases beyond pH 6.5. It was expected that ΔC_H would be zero at all pH ranges above 6.0, if not negative because of the formation of hydroxy complexes. This illustrates one of the limitations of using the delta quantities with experimental data. ΔC_H was calculated on the basis of the ligand protonation constants, total ligand in solution and the experimentally measured C_H. ΔC_H may therefore suffer from many errors. First, the [L] and [M] data have been calculated with a $p[L]_o$ and $p[M]_o$ which may be in error, second, the ligand protonation constants may be in error and, finally, the C_H values derived from a titration curve may be less than ideal since in the particular titration chosen C_L was maintained constant while C_M was allowed to vary through dilution. As was discussed in Chapter II, during the experiment only one of the components needed to be held constant. C_L and C_M used to calculate ΔC_L and ΔC_M values were fixed at the values of the middle titration, but the value of C_H varies through the titration and must therefore be taken from actual experimental data. The C_H values are also limited by the need to use a hydrogen ion concentration and, at extremes of pH this could contribute a large error to the ΔC_H values if the pH is converted directly to $[H^+]$. However, the delta quantities were introduced for the purpose of aiding in the selection of a model and approximate values are generally sufficient for this purpose.

The rise in ΔC_H at the ends of the pH range, shown in Figure 21, is most likely a result of the errors just discussed. A rise in ΔC_H at high pH is unlikely in the zinc-aspartic acid system because there are no known protonated complexes at high pH. If some error existed in the protonation constant for HL the effect of that error would decrease at higher pH where HL is being titrated to L.

Figure 22 shows the ΔC_H, ΔC_L and ΔC_M curves calculated on the basis of the [L] and [M] data discussed above, and estimated constants for the species H_2L, HL, HML and ML. The values of ΔC_H, ΔC_L and ΔC_M have essentially been reduced to zero from pH 4.0 to pH 6.5. The complex forming above pH 6.5 is ML_2.

Figure 23 shows ΔC_H, ΔC_L and ΔC_M calculated using constants for H_2L, HL, HML, ML and ML_2. There is a slight rise in ΔC_L and ΔC_M above pH 8.5, indicating a complex ML_3. As discussed above, ΔC_H has sufficient error associated with it that it should not be used as the basis of a search for protonated complexes above pH 7.0. The value of ΔC_H can be made more reliable with better sets of [L] and [M] data and better values for the ligand protonation constants. It should be recalled that one advantage of the FICS method is that it does not attempt to explicitly fit the C_H data, but rather fitting the ligand and metal mass

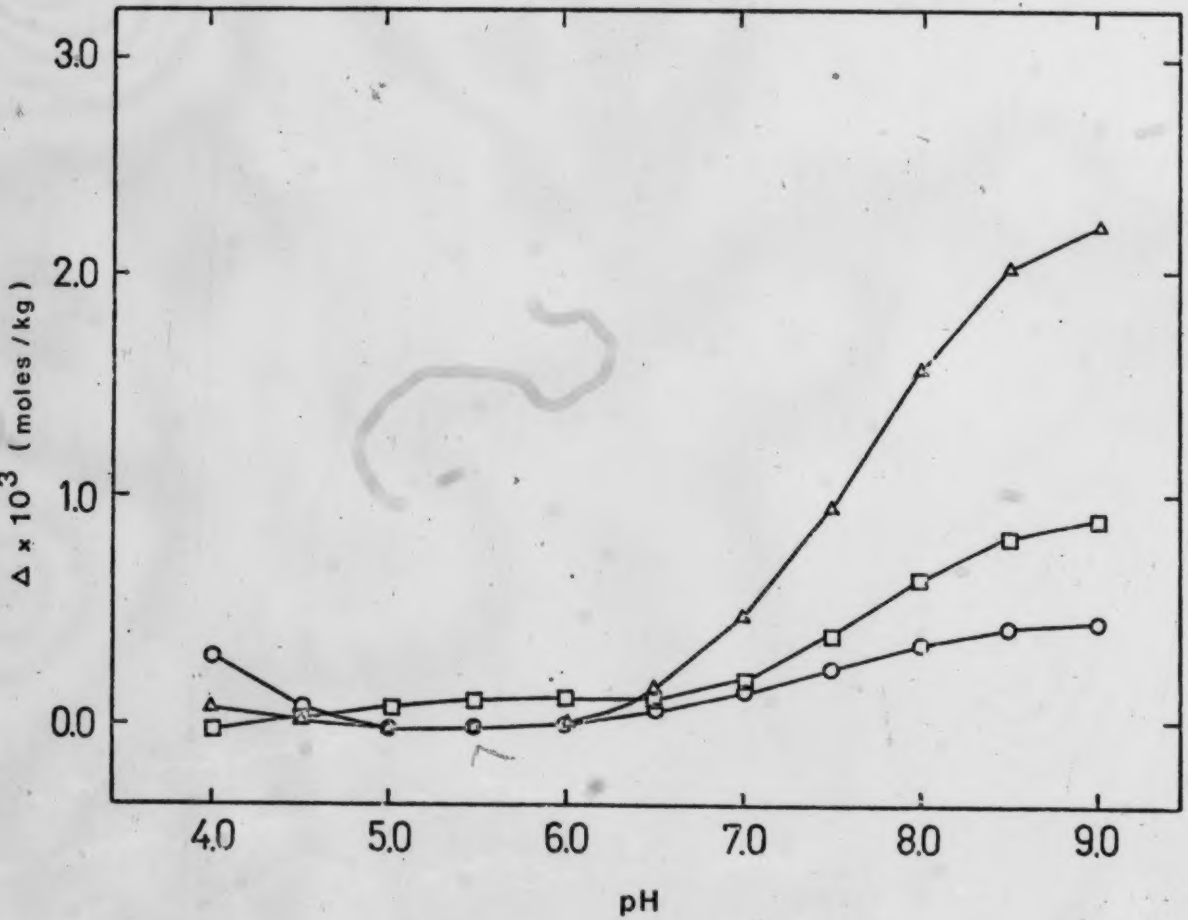


Figure 22. ΔC_H, ○ ; ΔC_L, △ ;and ΔC_M, □ ; as in Figure 21, except that estimates of the formation constants for HML and ML have been used to calculate the delta quantites.

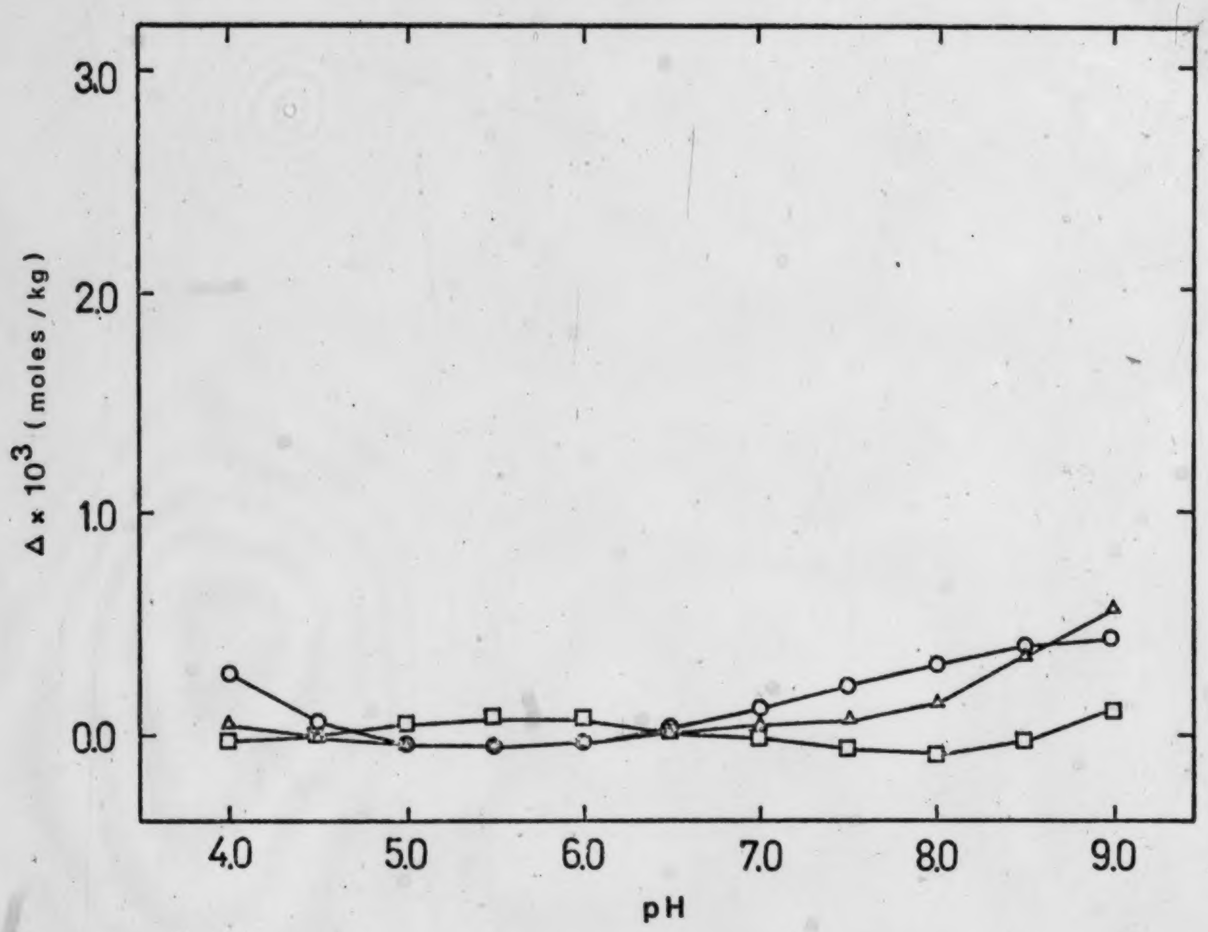


Figure 23. ΔC_H, o; ΔC_L, ▵ ;and ΔC_M, ▫ ; as in Figure 22, except that estimates of the formation constants for HML, ML and ML_2 have been used to calculate the delta quantities.

balances is considered more important at this point in the calculations.

The next stage in the FICS calculation procedure is the search for good estimates of $p[L]_o$ and $p[M]_o$ and the calculation of formation constants. Since the major complexation begins at pH 5.0 this was chosen as pH_o, and the initial free ligand and metal at this point was found. Initially, it was assumed that no complex formed, and $p[L]_o$ was calculated to be 6.890 and $p[M]_o$ as 3.030. With these values and the functions (dC_H/dC_L) and (dC_H/dC_M), [L] and [M] from pH 5.0 to 8.0 were calculated. Assuming the existence of HML, ML and ML_2, constants were calculated from the ligand mass balance,the metal mass balance and both mass balances together. The constants on $\sum L$ were unreasonable, and a negative constant was found for $\sum M$. The constants for both balances combined, $\sum ML$, were acceptable (good constants for ligand protonation). The concentrations of HML and ML were calculated and the initial free ligand and free metal concentrations were adjusted accordingly. A new set of [L] and [M] values were calculated, also a new set of formation constants. After three such iterations, the value for HML based on $\sum M$ was no longer negative. The iteration was carried out 6 times, after which the values of $p[L]_o$ no longer changed and $p[M]_o$ only changed by 0.002 units. The constants changed only slightly with the last iteration. During this iteration the values of $p[L]_o$ and $p[M]_o$ changed less and less with each repeat of the procedure. For example $p[M]_o$ varied as 3.030, 3.037, 3.043, 3.048, 3.052, 3.055 and 3.057. This sequence indicates that $p[M]_o$ was 3.057 ± 0.003 at pH 5.0. Unlike the sequence described to give the acid dissociation constants for aspartic acid, this sequence does not appear likely to produce a new estimate of $p[L]_o$ and $p[M]_o$ exactly the same as the previous one. In this case the iteration can only be carried on until the changes are as small as can be justified by the expected accuracy of the formation constants.

Table 29 shows formation constants derived with the data above pH 5.0. Two alternatives are shown, the first in which HML is not included in the model of complexation and the second in which it is considered. In the first case iteration stopped immediately because very little ML existed at pH 5.0. The second set of constants is the final result of the series of 6 iterations described above. It is of interest to note that the constants for ML and ML_2, calculated on the basis of these two models, are almost identical. There is insufficient evidence to prove that HML actually exists in solution. First, the formation constant for HML is small and, second, its inclusion does not significantly change either the standard deviation of the fit or the values of formation constants of the other species in solution. A species distribution for the model including ML and ML_2 is shown in Figure 24.

The FICS method would be most convenient if it was possible to begin at a pH_o at which no complexation had

Table 29. Determination of the Formation Constants of Complexes of Zinc and Aspartic Acid with pH_o 5.0

Species	log (β)		
	ΣL	ΣM	ΣML
HL[a]	9.60		9.59
ML	5.60	5.69	5.66
ML_2	10.16	10.05	10.14
SD of fit:	0.96%	1.76%	SD_L=1.08% SD_M=4.26%
HL[b]	9.59		9.59
HML		10.52	10.76
ML	5.62	5.73	5.68
ML_2	10.18	10.06	10.17
SD of fit:	0.96%	1.49%	SD_L=1.02% SD_M=4.74%

[a] $p[L]_o$= 6.890, $p[M]_o$= 3.030, on the basis of no complexation at pH_o= 5.0. Range of data: pH 5.0 - 8.0.

[b] $p[L]_o$ and $p[M]_o$ calculated by the iterative procedure described in the text. $p[L]_o$= 6.887, $p[M]_o$= 3.057 at pH_o= 5.0. Range of data: pH 5.0 - 8.0.

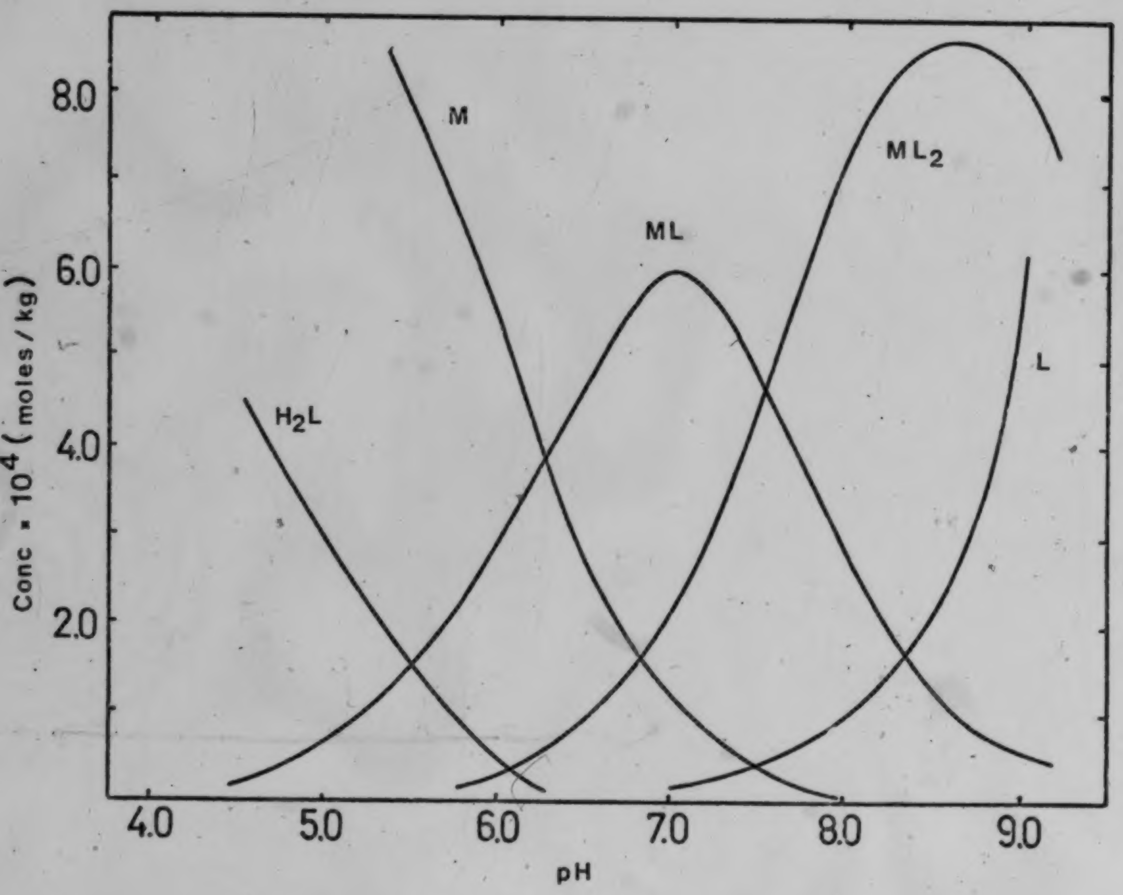


Figure 24. Species distribution as a function of pH for a solution containing 9.370×10^{-4} moles/kg of zinc and 5.308×10^{-3} moles/kg of aspartic acid.

occurred and where $p[L]_o$ and $p[M]_o$ could be calculated easily. This cannot be done because the values of (dC_H/dC_L) and (dC_H/dC_M) become less reliable at extremes of the pH range, and these are usually the only regions where complexation may be absent. In addition to the problems caused by the uncertainty in C_H at extremes of pH, a second problem became evident during the zinc-aspartic acid experiments.

At pH 4.0 very little complexation occurred. This could not be considered an extreme pH, yet the reliability of (dC_H/dC_M) is quite low. This is because the titration curves were very similar in that pH range and, consequently, the ΔC_H term in $(\Delta C_H/\Delta C_M)$ was small. The difference in the titration curves caused by the normal errors was larger than the effect of significance, namely the effect of complexation. Integration over a set of (dC_H/dC_M) in which no complexation is occurring only accumulates the errors in those derivatives and provides almost no information about complexation. The most reliable data appears to be obtained if more than 10% of the metal (conditions of large excess of ligand) is complexed.

Table 30 summarizes a number of the protonation constants of aspartic acid and formation constants of complexes between aspartic acid and zinc in the literature. Martell and Smith (60) consider that the best reported values for the formation constants of the species H_2L and HL are 13.33 and 9.63 respectively. The best values reported

Table 30. The Protonation Constants for Aspartic Acid, and the Stability Constants of Zinc-Aspartic Acid Complexes Reported in the Literature.

log (β)				Conditions	Ref
H_2L	HL	ML	ML_2	Temp, I	
13.33	9.63			25°C, 0.1	60
13.29	9.62			20°C, 0.1	60
		5.84	10.15	30°C, 0.1	61
13.74	9.87			25°C, 0.1	62
13.42	9.63			30°C, 0.1	63
	9.59			30°C, 1.0	64
13.34	9.63			25°C, 0.1	65
			10.4	15°C, 0.04	66
13.34	9.56			20°C, 1.0	67
	9.56			20°C, 1.0	68
13.32	9.62			25°C, 0.1	69
13.14	9.46			30°C, 0.1	61
13.92	9.98			20°C, 0.01	66
13.25	9.60			20°C, 0.1	70
13.31	9.61			25°C, 1.0	71

in this work are 13.356 and 9.678 respectively, found in Table 27. The ligand protonation constants determined simultaneously with stability constants, shown in Tables 28 and 29, also agree well with those in the literature. The constants for ML and ML_2 reported by Chaberek and Martell in reference 61 were 5.84 and 10.15. The constants for those species, shown in Table 28, are in good agreement with these values. The constants for ML appearing in Table 29 are about 0.2 log units smaller than those in Table 28, while the constants for ML_2 are similar to those in Table 28.

The stability constants for the model including ML and ML_2, shown in Table 29, were used to simulate a titration curve which had been obtained experimentally. The curves are shown in Figure 25. The agreement between these curves is very good, especially in the region used to calculate the formation constants of ML and ML_2.

D. Summary.

Many of the calculation techniques described in Chapter II were applied to data collected to study the complexation of aspartic acid to zinc. It was observed that there were no sharp discontinuities in the curves of C_H vs C_L. The onset of precipitation may have been responsible for this. A series of titrations were performed at a 5:1 ligand to metal ratio, and each component was varied about 10%. It

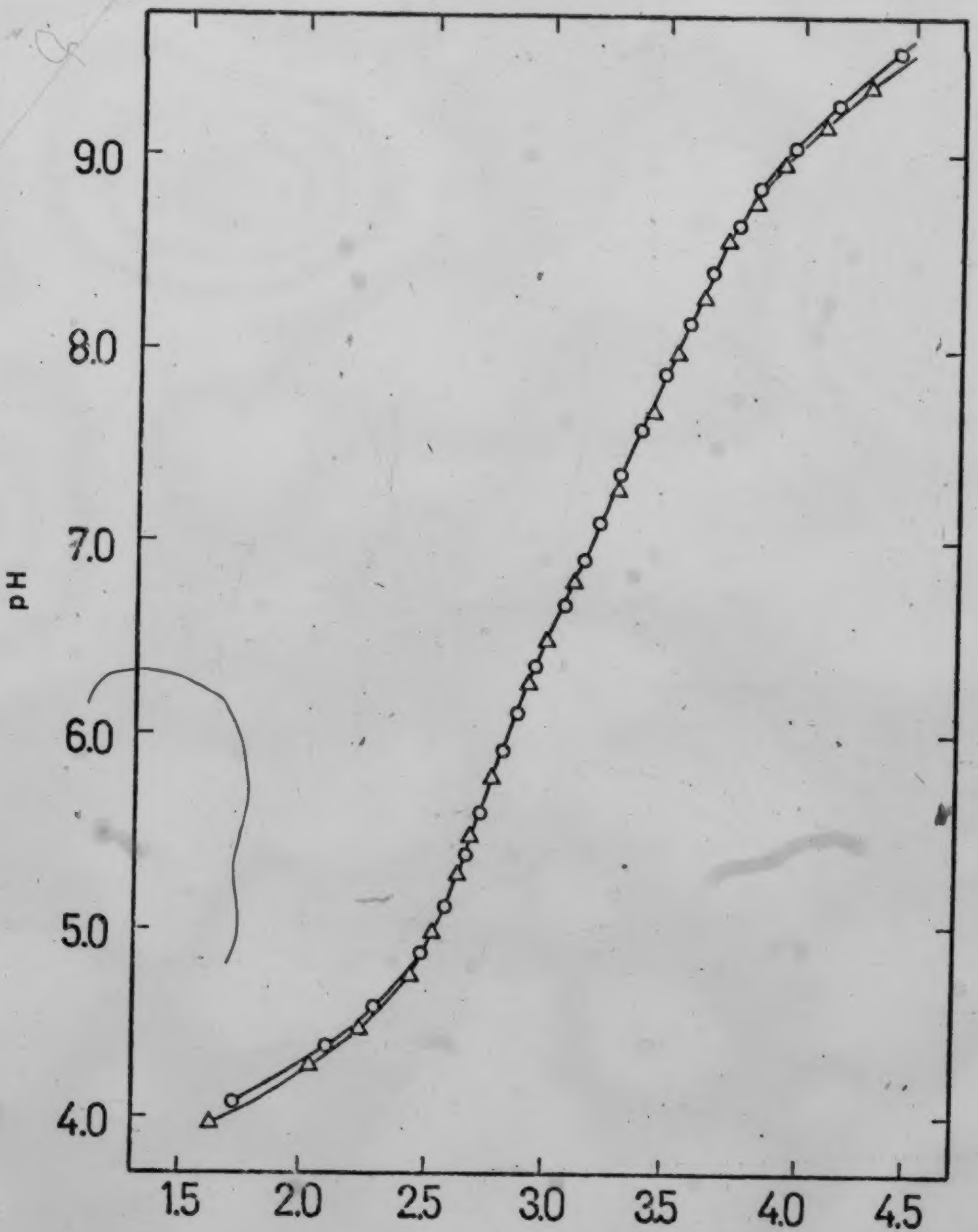


Figure 25. Experimental, o, and simulated, ▵, titration curves of a solution containing 9.370 x 10^{-4} moles/kg of zinc and 5.308 x 10^{-3} moles/kg of aspartic acid. The titration was simulated using a model of complexation including the species ML and ML_2 (See Table 29).

was observed that below pH 5.0 only slight complexation of aspartic acid to zinc was occurring and as a result the values of (dC_H/dC_M) became less reliable. This difficulty can in part be avoided by using a wider range of C_L and C_M, especially if $C_L \gg C_M$. The series of titrations used to study the complexation of glutathione, described in the next chapter, were run at less of an excess of ligand and a wider variation in C_L and C_M, both corrections designed to keep ΔC_H significant over as wide a range of pH as possible.

The methods used to find the best estimate of $p[L]_o$ and $p[M]_o$ were found to work in the manner described in Chapter II. It was found however, that there were many situations where this procedure would fail. For example, if too wide a pH range or a pH range containing too many complexes was chosen, the $p[L]_o$ and $p[M]_o$ could change continuously beyond values that would be considered reasonable. This will occur if, within each iteration, $p[L]_o$ and $p[M]_o$ change in the direction of more complexation. As a result the values of each set of new formation constants is larger, and the new $p[L]_o$ and $p[M]_o$ must be calculated assuming even more complexation. If the data is good and the model of complexation appropriate, this problem should in general be avoidable. In the discussion of the complexation of glutathione to zinc, in the following chapter, an alternative method of finding $p[L]_o$ and $p[M]_o$ based on a trial and error search for a minimum in standard deviation is described. There is no reason to believe one method is better than the other and, if the data is good, both should give reasonable, if not identical, values of $p[L]_o$ and $p[M]_o$.

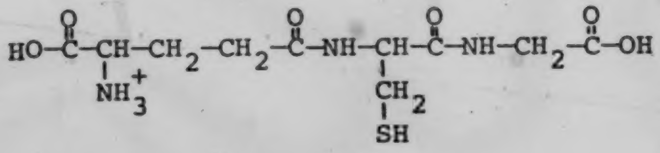
Finally, it was found that the protonation constants and complex formation constants derived with the various mass balances and different values of $p[L]_o$ and $p[M]_o$ were rather insensitive to those changes, as may be seen in the constants for ML_2 shown in the various parts of Tables 28 and 29. This is a good indication of the reliability of that constant. The constants for species found in low concentrations and at the extremes of pH, and for the other species which coexist with those in some regions of pH, may be somewhat less reliable. The final test, as described in Chapter II, is the simulation of a titration using the constants derived from the FICS method and comparing this titration to actual experimental results. Good agreement between these is expected if the model for complexation and the stability constants are correct.

CHAPTER VI

THE COMPLEXATION OF ZINC, CADMIUM AND LEAD BY GLUTATHIONE

A. Introduction.

Glutathione is a tripeptide composed of glutamic acid, cysteine and glycine residues.

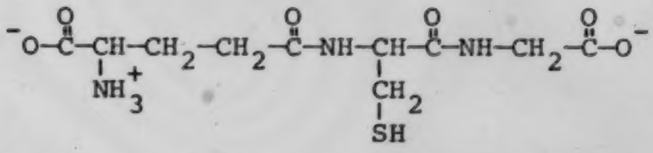


$$\mathrm{HO{-}\overset{O}{\overset{\|}{C}}{-}\underset{\underset{NH_3^+}{|}}{CH}{-}CH_2{-}CH_2{-}\overset{O}{\overset{\|}{C}}{-}NH{-}\underset{\underset{\underset{SH}{|}}{CH_2}}{\underset{|}{CH}}{-}\overset{O}{\overset{\|}{C}}{-}NH{-}CH_2{-}\overset{O}{\overset{\|}{C}}{-}OH}$$

The amino and carboxyl groups of the glutamic acid residue, the sulfhydryl group of cysteine and the carboxyl group of the glycine residue have ionizable protons and are the groups most likely to participate in coordination to metal ions. At pH's above 12.0 protons on the peptide linkages may also be removed.

The acid-base behavior of glutathione must be described in terms of a series of microscopic ionizations. At low pH all four groups are protonated and a single form of H_4L exists in solution. Near pH 2.0 a carboxyl proton from either the glutamyl or glycine group may be removed, giving two possible coexisting species with the stoichiometry H_3L. Since the glutamyl carboxyl is slightly more acidic, the species deprotonated at that site predominates; however, both do exist at detectable concentrations (72).

In the pH range from 3.0 to 4.0 the second proton is removed and a single species H_2L exists in solution.



$$^{-}O-\overset{O}{\overset{\|}{C}}-\underset{NH_3^+}{\underset{|}{CH}}-CH_2-CH_2-\overset{O}{\overset{\|}{C}}-NH-\underset{\underset{SH}{|}}{\underset{CH_2}{\underset{|}{CH}}}-\overset{O}{\overset{\|}{C}}-NH-CH_2-\overset{O}{\overset{\|}{C}}-O^{-}$$

Above pH 8.0 the process repeats itself, the amino and sulfhydryl groups losing protons simultaneously. The sulfhydryl is slightly more acidic and as a result the species having that group deprotonated is the predominant form of HL. The final proton is removed above pH 9.5.

Glutathione may bind to metal ions through the four groups discussed above. Most transition metals and "soft" metals such as mercury bind predominantly through the sulfhydryl group, often displacing the sulfhydryl proton at low pH. The other groups of the molecule may be protonated, deprotonated, bound to the same metal ion as the sulfhydryl or bound to another metal ion. This could potentially lead to an extremely complicated mixture of species existing in solution.

In this chapter a study of the interactions of glutathione with zinc, cadmium and lead using the FICS method is described. The pH titrations were performed using the optical couple gravimetric apparatus described in Chapters III and IV, and the data was evaluated using the methods developed in Chapter II. The discussion will be divided into two sections, the first dealing with the experimental results, the application of the FICS method and the deduction of species present in solution, and the second dealing in more detail with the chemistry involved, the structure of the complexes and a comparison of the results with those of other workers.

B. Results.

Determination of the Acid Dissociation Constants of Glutathione

A series of solutions containing from 1.928 x 10^{-3} to 5.301 x 10^{-3} moles/kg of glutathione were titrated from pH 3.3 to 10.9. Details of the composition of these samples is shown in Table 31. The concentration of titratable protons, C_H, was calculated at each 0.1 pH unit using the method of interpolation described in Chapter II. At each point the change in C_H with total glutathione concentration was calculated, giving (dC_H/dC_L) as a function of pH. The values of C_H and (dC_H/dC_L) are summarized at 0.5 intervals on Tables 32 and 33.

The concentration of deprotonated glutathione, [L], was calculated using Equation 9 and the values of (dC_H/dC_L) are shown in Table 33. Since the initial free glutathione was not known at any pH, a series of estimates were tried at pH 10.5. High pH was chosen because in this region [L]

Table 31. Determination of the Acid Dissociation Constants of Glutathione: Solution Composition Data.

Sample	Weight Glutathione[a] gm	Solvent Weight[b] gm	Concentration of Added Acid moles/kg	Concentration of Glutathione moles/kg
1	10.002	190.002	1.101×10^{-4}	4.819×10^{-3}
2	8.000	192.003	1.113×10^{-4}	3.855×10^{-3}
3	4.000	195.998	1.136×10^{-4}	1.928×10^{-3}
4	11.001	189.002	1.095×10^{-4}	5.301×10^{-3}
5	6.003	194.004	1.124×10^{-4}	2.891×10^{-3}

[a] The glutathione concentration was 0.09636 moles/kg.

[b] The solvent contained 1.159×10^{-4} moles/kg of HNO_3.

Table 32. Determination of the Acid Dissociation Constants of Glutathione: C_H data.

	C_H, moles/kg				
	Glutathione Concentration, moles/kg				
pH	1.928×10^{-3}	2.891×10^{-3}	3.855×10^{-3}	4.819×10^{-3}	5.301×10^{-3}
3.5	4.994×10^{-3}	7.364×10^{-3}	1.006×10^{-2}	1.213×10^{-2}	1.330×10^{-2}
4.0	4.256×10^{-3}	6.365×10^{-3}	8.462×10^{-3}	1.056×10^{-2}	1.162×10^{-2}
4.5	3.926×10^{-3}	5.896×10^{-3}	7.862×10^{-3}	9.820×10^{-3}	1.080×10^{-2}
5.0	3.795×10^{-3}	5.716×10^{-3}	7.626×10^{-3}	9.532×10^{-3}	1.050×10^{-2}
5.5	3.750×10^{-3}	5.650×10^{-3}	7.546×10^{-3}	9.430×10^{-3}	1.038×10^{-2}
6.0	3.726×10^{-3}	5.619×10^{-3}	7.505×10^{-3}	9.385×10^{-3}	1.032×10^{-2}
6.5	3.704×10^{-3}	5.592×10^{-3}	7.470×10^{-3}	9.346×10^{-3}	1.027×10^{-2}
7.0	3.671×10^{-3}	5.541×10^{-3}	7.404×10^{-3}	9.262×10^{-3}	1.018×10^{-2}
7.5	3.582×10^{-3}	5.404×10^{-3}	7.219×10^{-3}	9.035×10^{-3}	9.927×10^{-3}
8.0	3.335×10^{-3}	5.037×10^{-3}	6.715×10^{-3}	8.425×10^{-3}	9.244×10^{-3}
8.5	2.781×10^{-3}	4.212×10^{-3}	5.597×10^{-3}	7.061×10^{-3}	7.731×10^{-3}
9.0	1.895×10^{-3}	2.899×10^{-3}	3.847×10^{-3}	4.864×10^{-3}	5.344×10^{-3}
9.5	9.384×10^{-4}	1.498×10^{-3}	2.009×10^{-3}	2.580×10^{-3}	2.833×10^{-3}
10.0	1.937×10^{-4}	4.432×10^{-4}	6.628×10^{-4}	9.315×10^{-4}	1.015×10^{-3}
10.5	-4.611×10^{-4}	-3.536×10^{-4}	-2.649×10^{-4}	-1.602×10^{-4}	-1.588×10^{-4}

Table 33. Determination of the Acid Dissociation Constants of Glutathione: The Derivatives $(dC_H/dC_L)_{C_M,pH}$ and Calculated Free Ligand Concentration Using $p[L]_o$ of 3.040 at pH_o 10.5

pH	$(dC_H/dC_L)_{C_M,pH}$	[L]
3.5	2.492	3.76×10^{-15}
4.0	2.221	5.60×10^{-14}
4.5	2.082	6.61×10^{-13}
5.0	2.030	7.01×10^{-12}
5.5	2.010	7.16×10^{-11}
6.0	2.002	7.20×10^{-10}
6.5	1.994	7.19×10^{-9}
7.0	1.977	7.08×10^{-8}
7.5	1.929	6.74×10^{-7}
8.0	1.803	5.83×10^{-6}
8.5	1.521	4.02×10^{-5}
9.0	1.068	1.80×10^{-4}
9.5	0.5935	4.67×10^{-4}
10.0	0.2623	7.50×10^{-4}
10.5	0.0999	9.12×10^{-4}

would be nearly equal to C_L, thus simplifying a search for the best $p[L]_o$. With each $p[L]_o$ a set of [L] values was calculated, and with these sets of values, protonation constants of glutathione were calculated. The best values of $p[L]_o$ and the constants were chosen on the basis of the fit of the [L] data and constants to the total glutathione in solution. For a solution having a concentration of 1.0×10^{-3} moles/kg in glutathione, the best value of $p[L]_o$ at pH 10.5 was found to be 3.040. The [L] data calculated from this $p[L]_o$ are shown in Table 33 and the protonation constants for glutathione are shown in Table 34. These results are compared to those of other workers in Table 35.

The Determination of the Formation of Complexes Between Zinc and Glutathione

Two series of 5 titrations each were carried out on solutions containing varying concentrations of zinc and glutathione. The solution composition data for these titrations is shown in Table 36. The first set of five was designed to measure the change in C_H with changing concentrations of metal ion with constant ligand concentration. The concentrations of titratable proton for zinc concentrations from 7.820×10^{-4} to 1.173×10^{-3} moles/kg are shown as a function of pH in Table 37. As required by the FICS method, concentrated glutathione solution was added during the titration to maintain constant ligand

Table 34. The Acid Dissociation Constants of Glutathione.

Species	Overall Formation Constant, β	Log(β)	pK_a
H_4L	2.83×10^{23}	23.452	1.852
H_3L	3.975×10^{21}	21.600	3.460
H_2L	1.383×10^{18}	18.141	8.640
HL	3.170×10^{9}	9.501	9.501

Table 35. The Acid Dissociation Constants for Glutathione Reported in the Literature.

pK_a				Conditions	Ref
H_4L	H_3L	H_2L	HL	Temp, I	
2.05	3.40	8.72	9.49		72
		8.74	9.62	25°C, 0.16	73
	3.59	8.75	9.65	25°C, 0.15	48, 49
		8.96	9.35		50

Table 36. Solution Composition Data for the Zinc-Glutathione Experiments.

Sample	Concentration of Glutathione, moles/kg	Concentration of Zinc, moles/kg	Concentration of Added Acid, moles/kg
1	3.855×10^{-3}	9.775×10^{-4}	1.055×10^{-4}
2	3.855×10^{-3}	8.798×10^{-4}	1.060×10^{-4}
3	3.855×10^{-3}	1.075×10^{-3}	1.049×10^{-4}
4	3.855×10^{-3}	7.820×10^{-4}	1.066×10^{-4}
5	3.855×10^{-3}	1.173×10^{-3}	1.043×10^{-4}
6	3.855×10^{-3}	9.775×10^{-4}	1.055×10^{-4}
7	3.373×10^{-3}	9.775×10^{-4}	1.060×10^{-4}
8	4.337×10^{-3}	9.775×10^{-4}	1.049×10^{-4}
9	2.891×10^{-3}	9.775×10^{-4}	1.066×10^{-4}
10	4.819×10^{-3}	9.775×10^{-4}	1.043×10^{-4}

Table 37. C_H Data for the Zinc-Glutathione Experiments at Constant Glutathione Concentration.

	C_H, moles/kg				
	Zinc Concentration, moles/kg				
pH	7.820×10^{-4}	8.798×10^{-4}	9.775×10^{-4}	1.075×10^{-3}	1.173×10^{-3}
3.5	9.799×10^{-3}	9.879×10^{-3}	9.876×10^{-3}	9.787×10^{-3}	9.774×10^{-3}
4.0	8.546×10^{-3}	8.585×10^{-3}	8.636×10^{-3}	8.541×10^{-3}	8.538×10^{-3}
4.5	7.942×10^{-3}	7.957×10^{-3}	8.028×10^{-3}	7.938×10^{-3}	7.930×10^{-3}
5.0	7.672×10^{-3}	7.675×10^{-3}	7.753×10^{-3}	7.657×10^{-3}	7.648×10^{-3}
5.5	7.438×10^{-3}	7.426×10^{-3}	7.481×10^{-3}	7.364×10^{-3}	7.340×10^{-3}
6.0	6.926×10^{-3}	6.886×10^{-3}	6.873×10^{-3}	6.701×10^{-3}	6.637×10^{-3}
6.5	6.241×10^{-3}	6.122×10^{-3}	6.046×10^{-3}	5.802×10^{-3}	5.681×10^{-3}
7.0	5.752×10^{-3}	5.560×10^{-3}	5.445×10^{-3}	5.148×10^{-3}	4.968×10^{-3}
7.5	5.359×10^{-3}	5.137×10^{-3}	4.998×10^{-3}	4.670×10^{-3}	4.462×10^{-3}
8.0	4.874×10^{-3}	4.660×10^{-3}	4.507×10^{-3}	4.167×10^{-3}	3.954×10^{-3}
8.5	4.062×10^{-3}	3.900×10^{-3}	3.760×10^{-3}	3.431×10^{-3}	3.243×10^{-3}
9.0	2.773×10^{-3}	2.702×10^{-3}	2.596×10^{-3}	2.302×10^{-3}	2.164×10^{-3}
9.5	1.258×10^{-3}	1.242×10^{-3}	1.189×10^{-3}	9.171×10^{-4}	8.269×10^{-4}

concentration (3.855×10^{-3} moles/kg). The stock glutathione solution was used for this purpose (see Table 21). The quantity added during a typical titration was less than 0.5 gm.

The second set of titrations was performed at varying glutathione concentrations with the metal concentration constant. The C_H data for variation in glutathione from 2.891×10^{-3} to 4.819×10^{-3} moles/kg at constant zinc of 9.775×10^{-4} moles/kg is shown in Table 38.

The derivatives (dC_H/dC_L) and (dC_H/dC_M) were evaluated at 0.1 pH intervals from pH 5.5 to 9.0. During this step it became clear that some of the titrations deviated appreciably from the others. If the C_H values from three or four titrations lay on straight lines while the remainder deviated at every pH value, the deviating titrations were removed. In the case of the zinc-glutathione experiments two of five of each set were rejected at this point. Since these were in the middle of the ranges varied, this did not sacrifice accuracy of the (dC_H/dC_L) and (dC_H/dC_M) quantities. In the case of the cadmium and lead experiments described later no titrations were rejected. The final values of (dC_H/dC_L) and (dC_H/dC_M) are shown in Table 39. As this data indicates, only small amounts of metal are complexed at pH 5.5. It was therefore not necessary to begin the calculations at lower pH values. Only when (dC_H/dC_M) reaches values appreciably different from zero can they provide a reasonable quantity of information about the

Table 38. C_H Data for the Zinc-Glutathione Experiments at Constant Zinc Concentration.

	C_H, moles/kg				
	Glutathione Concentration, moles/kg				
pH	2.891×10^{-3}	3.373×10^{-3}	3.855×10^{-3}	4.337×10^{-3}	4.819×10^{-3}
3.5	7.361×10^{-3}	8.474×10^{-3}	9.314×10^{-3}	1.096×10^{-2}	1.199×10^{-2}
4.0	6.356×10^{-3}	7.378×10^{-3}	8.242×10^{-3}	9.536×10^{-3}	1.048×10^{-2}
4.5	5.880×10^{-3}	6.851×10^{-3}	7.748×10^{-3}	8.837×10^{-3}	9.758×10^{-3}
5.0	5.666×10^{-3}	6.608×10^{-3}	7.500×10^{-3}	8.522×10^{-3}	9.422×10^{-3}
5.5	5.460×10^{-3}	6.360×10^{-3}	7.160×10^{-3}	8.212×10^{-3}	9.060×10^{-3}
6.0	4.969×10^{-3}	5.772×10^{-3}	6.427×10^{-3}	7.544×10^{-3}	8.309×10^{-3}
6.5	4.147×10^{-3}	4.915×10^{-3}	5.600×10^{-3}	6.674×10^{-3}	7.466×10^{-3}
7.0	3.450×10^{-3}	4.265×10^{-3}	5.033×10^{-3}	6.050×10^{-3}	6.882×10^{-3}
7.5	2.986×10^{-3}	3.802×10^{-3}	4.574×10^{-3}	5.567×10^{-3}	6.394×10^{-3}
8.0	2.576×10^{-3}	3.343×10^{-3}	4.033×10^{-3}	5.026×10^{-3}	5.785×10^{-3}
8.5	2.061×10^{-3}	2.697×10^{-3}	3.179×10^{-3}	4.179×10^{-3}	4.766×10^{-3}
9.0	1.311×10^{-3}	1.734×10^{-3}	1.936×10^{-3}	2.857×10^{-3}	3.204×10^{-3}
9.5	2.866×10^{-4}	5.686×10^{-4}	5.571×10^{-4}	1.266×10^{-3}	1.405×10^{-3}

Table 39. The Derivatives $(dC_H/dC_L)_{C_M,pH}$ and $(dC_H/dC_M)_{C_L,pH}$ for the Zinc-Glutathione Experiments.

pH	$(dC_H/dC_L)_{C_M,pH}$	$(dC_H/dC_M)_{C_L,pH}$
5.5	1.930	-0.254
6.0	1.809	-0.760
6.5	1.802	-1.481
7.0	1.858	-2.075
7.5	1.847	-2.378
8.0	1.749	-2.442
8.5	1.497	-2.179
9.0	1.075	-1.627

complexation occurring in solution. If the calculations begin at a lower pH, the experimental and calculation errors introduce uncertainty into [M], yet provide no information about complexation. It is therefore advantageous to select pH ranges where the most information about complexation is available in order to offset, at least in part, the detrimental effects of error.

The final calculation of the FICS method provides the free ligand and free metal concentrations from (dC_H/dC_L) and (dC_H/dC_M). However, $p[L]_o$ and $p[M]_o$ must be known. The search for $p[L]_o$ and $p[M]_o$ may involve a trial and error process where a series of estimates of $p[L]_o$ and $p[M]_o$ values are chosen to try to minimize the standard deviation of the fit of the model of complexes to the total metal and ligand in solution. This may in theory pose some problem, since initially the model is unknown. However in practice it was found that the choice of $p[L]_o$ and $p[M]_o$ has no effect on the search for a model of equilibria in solution. No case was observed where a model was simultaneously accepted at one set of $p[L]_o$ and $p[M]_o$ and rejected with an alternative set. The model was searched for and found using $p[L]_o$ and $p[M]_o$ assuming some reasonable amount of complexation at pH_o and, with this model $p[L]_o$ and $p[M]_o$ were refined.

The process of refinement of $p[L]_o$ and $p[M]_o$ for the studies of the complexation of glutathione involved independent searches for the best values of $p[L]_o$ and $p[M]_o$.

It was found that the standard deviation of the fit to the metal mass balance, SD_M, was constant if $p[M]_o$ was constant and, similarly, SD_L was constant if $p[L]_o$ was constant. The minimum in SD_M and SD_L were therefore searched out independently. A model for the equilibria in solution was accepted only if a clear minimum in both the SD_M and SD_L could be found.

The formation constants of the complexes of zinc and glutathione are shown in Table 41. These constants were calculated with the [L] and [M] data shown in Table 40. The values of $p[L]_o$ and $p[M]_o$ were chosen to give the best fit to this model. The relative SD_M was 3.60% and SD_L was 0.94%. The constants reported were calculated using both the metal and the ligand mass balances, while each was used independently while searching for a model. This has been described in Chapter II. The constant for HL was given as 3.17×10^9 in order that calculations remain within the dynamic range of the computer. The constant for H_2L was calculated simultaneously with those for the metal complexes and was found to be 1.36×10^{18}. This agrees very well with 1.38×10^{18} calculated from the series of titrations involving glutathione alone (the previous section, see Table 34).

The deduction of the species present in the zinc-glutathione system proceeded in much the same way as described in Chapter II. This involved the calculation of ΔC_H, ΔC_L and ΔC_M, defined as the concentration of

Table 40. The Free Ligand and Free Metal Concentration for the Zinc-Glutathione Experiments with $p[L]_0 = 9.596$ and $p[M]_o = 3.156$ at pH_o 5.5

pH	Glutathione [L], moles/kg	Zinc [M], moles/kg
5.5	2.54×10^{-10}	6.98×10^{-4}
6.0	2.18×10^{-9}	4.04×10^{-4}
6.5	1.71×10^{-8}	1.12×10^{-4}
7.0	1.41×10^{-7}	1.40×10^{-5}
7.5	1.20×10^{-6}	1.06×10^{-6}
8.0	9.61×10^{-6}	6.39×10^{-8}
8.5	6.33×10^{-5}	4.32×10^{-9}
9.0	2.81×10^{-4}	4.74×10^{-10}

Table 41. The Formation Constants of Complexes of Zinc and Glutathione.[a]

Species	Formation Constant, β	Log(β)
M(HL)	3.15×10^{14}	14.50
$M(HL)_2$	1.48×10^{29}	29.17
M(HL)(L)	1.04×10^{22}	22.02
$M(L)_2$	1.16×10^{13}	13.06
$M(HL)_3$	3.25×10^{42}	42.51
$M(HL)_2(L)$	8.12×10^{33}	33.91

[a] The standard deviation of the fit to the metal and ligand mass balances are 3.60% and 0.94% respectively. The protonation constant for the H_2L was 1.36×10^{18}; the protonation constant for HL was given as 3.17×10^{9}.

protons, ligands and metal whose chemical situation has not been found. The magnitude of these quantites and the relationship among them gives reasonable indication of the concentrations and stoichiometry of species which may be in solution. Figure 26 shows ΔC_H, ΔC_L and ΔC_M for the zinc-glutathione system, using [L] and [M] from the FICS calculations and the ligand protonation constants evaluated in an earlier experiment. Below pH 6, the complexes appear to contain about equal numbers of protons, ligands and metal, suggesting the existence of M(HL) species. In the range of pH 6 to pH 7, the situation changes to a 2:1 ligand to metal ratio with the number of protons lying between 1 and 2. This indicates that a mixture of $M(HL)_2$ and M(HL)(L) may exist in that pH range. Finally, above pH 8 the number of ligands per metal is greater than 2 and the complexes are being rapidly deprotonated as indicated by a decrease in ΔC_H.

It was found that M(HL), $M(HL)_2$, M(HL)(L) and $M(HL)_3$ were major components in solution in the pH range studied. At the upper extreme of pH a series of possible species including $M(L)_2$ and $M(HL)_2(L)$ were suggested but,since the range of data is narrow, proof of their existence is not conclusive.

The middle titration used in the FICS method was simulated with the constants shown in Table 41. The actual experimental curve and the simulated curve agree very well to pH 8.5. This is considered the final and most reliable

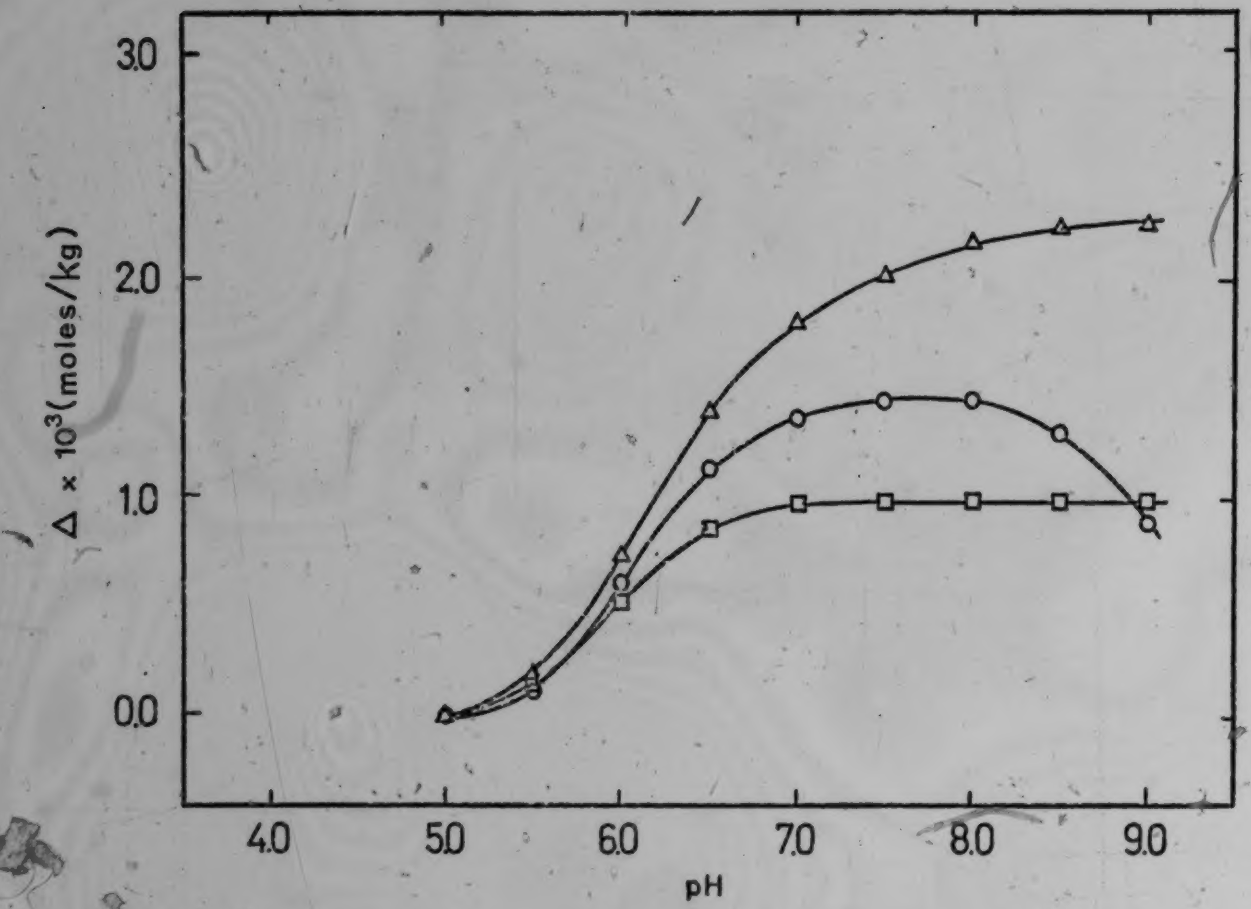


Figure 26. ΔC_H, o; ΔC_L, ▵; and ΔC_M, ▫; for the Zinc-Glutathione system.

indication of successful application of the FICS method to titration data. The constants were extracted from the ligand and metal mass balances and, for reasons described in Chapter II, not from the experimental C_H values. As a result, the model for complexation and the values of formation constants are not tailored to fit the C_H data. In comparison, other computer-based calculations,such as SCOGS, LETAGROP and so on, are specifically designed to fit C_H data. If, therefore, a titration is simulated using the model and constants from SCOGS, a good agreement with the experimental values of C_H is assured while, on the other hand, no such agreement is guaranteed using the FICS method. Good agreement will only occur if the model of complexation is correct and if the values of the formation constants are accurate.

Determination of the Formation Constants of Complexes Between Cadmium and Glutathione

Ten titrations were performed, five on solutions in which the concentration of glutathione was 3.907×10^{-3} moles/kg while the concentration of cadmium was varied from 5.820×10^{-4} to 1.358×10^{-3} moles/kg, and five on solutions in which the concentration of cadmium was held constant at 9.708×10^{-4} moles/kg while the concentration of glutathione was varied from 2.604×10^{-3} to 5.206×10^{-3} moles/kg. Details of the solution compositions are shown in Table 42.

Table 42. Solution Composition Data for the Cadmium-Glutathione Experiments.

Sample	Concentration of Glutathione, moles/kg	Concentration of Cadmium, moles/kg	Concentration of Added Acid, moles/kg
1	3.907×10^{-3}	9.698×10^{-4}	1.840×10^{-4}
2	3.907×10^{-3}	7.760×10^{-4}	1.860×10^{-4}
3	3.907×10^{-3}	1.164×10^{-3}	1.819×10^{-4}
4	3.910×10^{-3}	5.820×10^{-4}	1.880×10^{-4}
5	3.908×10^{-3}	1.358×10^{-3}	1.799×10^{-4}
6	3.905×10^{-3}	9.707×10^{-4}	1.840×10^{-4}
7	3.257×10^{-3}	9.708×10^{-4}	1.855×10^{-4}
8	4.532×10^{-3}	9.659×10^{-4}	1.826×10^{-4}
9	2.604×10^{-3}	9.708×10^{-4}	1.870×10^{-4}
10	5.206×10^{-3}	9.708×10^{-4}	1.809×10^{-4}

The concentrations of titratable protons for the series of titrations in which the cadmium concentration was varied and the concentrations of titratable protons for the series of titrations in which the glutathione concentration was varied are shown in Tables 43 and 44. Plots of C_H vs C_L and C_M revealed that no titrations deviated systematically from the expected straight lines and therefore all were used in the calculation of (dC_H/dC_L) and (dC_H/dC_M). The values for these derivatives are shown in Table 45.

The procedure used to determine the complexes existing in solution and the method to obtain the best values of $p[L]_o$ and $p[M]_o$ have already been described. The concentations of ligand and metal are shown in Table 46, and the constants for the formation of complexes between cadmium and glutathione are shown in Table 47.

The preliminary steps in the deduction of a model of cadmium-glutathione interactions required the calculation of ΔC_H, ΔC_L, and ΔC_M; these are shown on Figure 27. Because of inaccuracy in the value of $p[L]_o$ at this stage in the refinement of the data, the curve for ΔC_H appears to be too low by about 10×10^{-4}. There is also some uncertainty in ΔC_L and ΔC_M due to error in $p[L]_o$ and $p[M]_o$ but these errors will have no effect in later stages of calculations since ΔC_H, ΔC_L and ΔC_M were introduced to aid in the selection of a model. If the error becomes too large however, the delta quantities cannot be of much help in the selection of a model.

Table 43. C_H Data for the Cadmium-Glutathione Experiments Performed at Constant Glutathione Concentration.

	C_H, moles/kg				
	Cadmium Concentration, moles/kg				
pH	5.820×10^{-4}	7.760×10^{-4}	9.698×10^{-4}	1.164×10^{-3}	1.358×10^{-3}
3.5	9.95×10^{-3}	9.95×10^{-3}	9.96×10^{-3}	9.91×10^{-3}	9.91×10^{-3}
4.0	8.60×10^{-3}	8.61×10^{-3}	8.59×10^{-3}	8.55×10^{-3}	8.53×10^{-3}
4.5	7.88×10^{-3}	7.83×10^{-3}	7.75×10^{-3}	7.67×10^{-3}	7.56×10^{-3}
5.0	7.31×10^{-3}	7.12×10^{-3}	6.93×10^{-3}	6.76×10^{-3}	6.54×10^{-3}
5.5	6.90×10^{-3}	6.64×10^{-3}	6.39×10^{-3}	6.15×10^{-3}	5.91×10^{-3}
6.0	6.66×10^{-3}	6.35×10^{-3}	6.08×10^{-3}	5.78×10^{-3}	5.52×10^{-3}
6.5	6.45×10^{-3}	6.11×10^{-3}	5.82×10^{-3}	5.48×10^{-3}	5.20×10^{-3}
7.0	6.19×10^{-3}	5.83×10^{-3}	5.50×10^{-3}	5.15×10^{-3}	4.85×10^{-3}
7.5	5.84×10^{-3}	5.44×10^{-3}	5.09×10^{-3}	4.73×10^{-3}	4.41×10^{-3}
8.0	5.38×10^{-3}	4.94×10^{-3}	4.58×10^{-3}	4.21×10^{-3}	3.89×10^{-3}
8.5	4.59×10^{-3}	4.23×10^{-3}	3.92×10^{-3}	3.57×10^{-3}	3.26×10^{-3}
9.0	3.36×10^{-3}	3.15×10^{-3}	2.96×10^{-3}	2.66×10^{-3}	2.41×10^{-3}

Table 44. C_H Data for the Cadmium-Glutathione Experiments Performed at Constant Cadmium Concentration.

	C_H, moles/kg				
	Glutathione Concentration, moles/kg				
pH	2.604×10^{-3}	3.257×10^{-3}	3.905×10^{-3}	4.532×10^{-3}	5.206×10^{-3}
3.5	6.67×10^{-3}	8.29×10^{-3}	9.84×10^{-3}	1.143×10^{-3}	1.312×10^{-3}
4.0	5.67×10^{-3}	7.10×10^{-3}	8.47×10^{-3}	9.85×10^{-3}	1.136×10^{-2}
4.5	5.10×10^{-3}	6.40×10^{-3}	7.63×10^{-3}	8.85×10^{-3}	1.019×10^{-2}
5.0	4.48×10^{-3}	5.64×10^{-3}	6.78×10^{-3}	7.94×10^{-3}	9.23×10^{-3}
5.5	3.90×10^{-3}	5.06×10^{-3}	6.23×10^{-3}	7.40×10^{-3}	8.69×10^{-3}
6.0	3.53×10^{-3}	4.71×10^{-3}	5.89×10^{-3}	7.08×10^{-3}	8.35×10^{-3}
6.5	3.26×10^{-3}	4.44×10^{-3}	5.62×10^{-3}	6.79×10^{-3}	8.04×10^{-3}
7.0	3.00×10^{-3}	4.15×10^{-3}	5.30×10^{-3}	6.44×10^{-3}	7.64×10^{-3}
7.5	2.70×10^{-3}	3.79×10^{-3}	4.88×10^{-3}	5.96×10^{-3}	7.12×10^{-3}
8.0	2.32×10^{-3}	3.34×10^{-3}	4.36×10^{-3}	5.38×10^{-3}	6.46×10^{-3}
8.5	1.89×10^{-3}	2.80×10^{-3}	3.70×10^{-3}	4.59×10^{-3}	5.51×10^{-3}
9.0	1.32×10^{-3}	2.04×10^{-3}	2.74×10^{-3}	3.44×10^{-3}	4.09×10^{-3}

Table 45. The Derivatives $(dC_H/dC_L)_{C_M,pH}$ and $(dC_H/dC_M)_{C_L,pH}$ for the Cadmium-Glutathione Experiments.

pH	$(dC_H/dC_L)_{C_M,pH}$	$(dC_H/dC_M)_{C_L,pH}$
3.5	2.506	-0.059
4.0	2.224	-0.103
4.5	2.000	-0.423
5.0	1.876	-0.994
5.5	1.897	-1.297
6.0	1.915	-1.508
6.5	1.900	-1.661
7.0	1.850	-1.777
7.5	1.764	-1.891
8.0	1.658	-1.974
8.5	1.460	-1.770
9.0	1.130	-1.276

Table 46. The Free Ligand and Free Metal Concentrations for the Cadmium-Glutathione Experiments with $p[L]_o = 12.679$ and $p[M]_o = 3.080$ at pH_o 4.0

pH	Glutathione [L], moles/kg	Cadmium [M], moles/kg
4.0	2.09×10^{-13}	8.32×10^{-4}
4.5	2.38×10^{-12}	6.50×10^{-4}
5.0	2.17×10^{-11}	2.78×10^{-4}
5.5	1.90×10^{-10}	7.35×10^{-5}
6.0	1.71×10^{-9}	1.45×10^{-5}
6.5	1.54×10^{-8}	2.34×10^{-6}
7.0	1.34×10^{-7}	3.23×10^{-7}
7.5	1.07×10^{-6}	3.90×10^{-8}
8.0	7.75×10^{-6}	4.16×10^{-9}
8.5	4.72×10^{-5}	4.67×10^{-10}
9.0	2.12×10^{-4}	7.97×10^{-11}

Table 47. The Formation Constants of Complexes of Cadmium and Glutathione.[a]

Species	Formation Constant, β	Log(β)
$M(HL)$	4.63×10^{15}	15.66
$M(HL)_2$	1.16×10^{31}	31.06
$M(HL)M(HL)_2$	1.60×10^{50}	50.20
$M(HL)(L)$	5.02×10^{21}	21.70
$M(HL)_3$	2.57×10^{44}	44.41
$M(HL)_2(L)$	8.86×10^{35}	35.95

[a] The standard deviation of the fit of the metal and ligand mass balances are 1.79% and 0.46% respectively. The protonation constants for H_3L and H_2L were determined to be 5.20×10^{21} and 1.31×10^{18} respectively; the protonation constant for HL was given as 3.17×10^{9}.

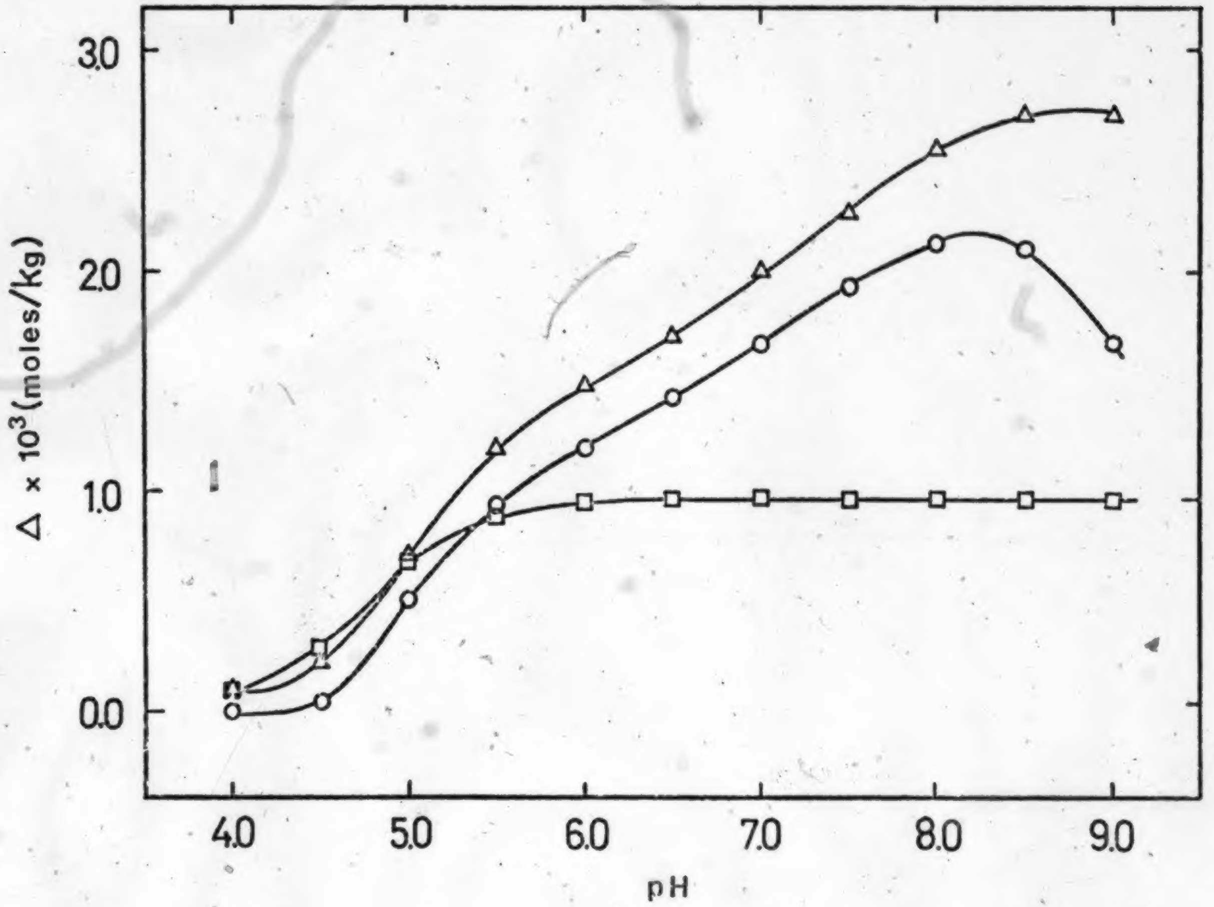


Figure 27. ΔC_H, ○; ΔC_L, △; and ΔC_M, □; for the Cadmium-Glutathione system.

In the range of pH 4.0 to 5.0 the predominant complex appears to be M(HL). In the range of pH 6.0 to 7.0 there are two ligands and two protons per metal, suggesting $M(HL)_2$. From pH 7.0 to 8.0 more than two ligands and two protons are complexed to each metal indicating that a mixture of $M(HL)_2$ and $M(HL)_3$ has formed. In the range of pH 8.0 to 9.0 the number of ligands per metal continues to increase, but the ΔC_H value drops rapidly above pH 8.3. The ligand appears to remain bound to cadmium exclusively as (HL) until pH 7.5. The major components of the cadmium-glutathione system were found to be M(HL), $M(HL)_2$ and $M(HL)_3$.

With the constants shown in Table 47 the middle titration was simulated and compared to the experimental C_H data. A small deviation was observed in the pH range of 6.5 to 8.0. This will be considered in the discussion section of this chapter.

Determination of the Formation Constants of Complexes Between Lead and Glutathione

Ten titrations were performed, five with varying metal and five with varying ligand concentrations. The details of the solution compositions are shown in Table 48. The C_H data for those titrations in which the metal concentration was varied appears in Table 49 and the C_H for those titrations in which the ligand concentration was varied appears in Table 50. Plots of C_H vs C_M and C_L indicated

Table 48. Solution Composition Data for the Lead-Glutathione Experiments.

Sample	Concentration of Glutathione, moles/kg	Concentration of Lead, moles/kg	Concentration of Added Acid, moles/kg
1	3.907×10^{-3}	1.016×10^{-3}	1.840×10^{-4}
2	3.906×10^{-3}	8.128×10^{-4}	1.860×10^{-4}
3	3.908×10^{-3}	1.219×10^{-3}	1.819×10^{-4}
4	3.907×10^{-3}	6.097×10^{-4}	1.880×10^{-4}
5	3.907×10^{-3}	1.423×10^{-3}	1.799×10^{-4}
6	3.908×10^{-3}	1.016×10^{-3}	1.840×10^{-4}
7	3.253×10^{-3}	1.016×10^{-3}	1.855×10^{-4}
8	4.510×10^{-3}	1.017×10^{-3}	1.826×10^{-4}
9	2.607×10^{-3}	1.016×10^{-3}	1.870×10^{-4}
10	5.206×10^{-3}	1.016×10^{-3}	1.809×10^{-4}

Table 49. C_H Data for the Lead-Glutathione Experiments Performed at Constant Glutathione Concentration.

	C_H, moles/kg				
	Lead Concentration, moles/kg				
pH	6.097×10^{-4}	8.128×10^{-4}	1.016×10^{-3}	1.219×10^{-3}	1.423×10^{-3}
3.5	9.94×10^{-3}	9.90×10^{-3}	9.91×10^{-3}	9.87×10^{-3}	9.86×10^{-3}
4.0	8.60×10^{-3}	8.55×10^{-3}	8.54×10^{-3}	8.47×10^{-3}	8.43×10^{-3}
4.5	7.80×10^{-3}	7.69×10^{-3}	7.62×10^{-3}	7.47×10^{-3}	7.36×10^{-3}
5.0	7.28×10^{-3}	7.09×10^{-3}	6.94×10^{-3}	6.73×10^{-3}	6.56×10^{-3}
5.5	6.96×10^{-3}	6.72×10^{-3}	6.51×10^{-3}	6.28×10^{-3}	6.06×10^{-3}
6.0	6.74×10^{-3}	6.47×10^{-3}	6.22×10^{-3}	5.95×10^{-3}	5.70×10^{-3}
6.5	6.55×10^{-3}	6.24×10^{-3}	5.96×10^{-3}	5.66×10^{-3}	5.39×10^{-3}
7.0	6.32×10^{-3}	5.98×10^{-3}	5.68×10^{-3}	5.35×10^{-3}	5.06×10^{-3}
7.5	6.00×10^{-3}	5.62×10^{-3}	5.30×10^{-3}	4.95×10^{-3}	4.66×10^{-3}
8.0	5.53×10^{-3}	5.12×10^{-3}	4.80×10^{-3}	4.45×10^{-3}	4.18×10^{-3}
8.5	4.74×10^{-3}	4.38×10^{-3}	4.12×10^{-3}	3.78×10^{-3}	3.54×10^{-3}
9.0	3.47×10^{-3}	3.22×10^{-3}	3.10×10^{-3}	2.82×10^{-3}	2.62×10^{-3}

Table 50. C_H Data for the Lead-Glutathione Experiments Performed at Constant Lead Concentration.

	C_H, moles/kg				
	Glutathione Concentration, moles/kg				
pH	2.607×10^{-3}	3.253×10^{-3}	3.908×10^{-3}	4.510×10^{-3}	5.206×10^{-3}
3.5	6.63×10^{-3}	8.22×10^{-3}	9.79×10^{-3}	1.13×10^{-2}	1.30×10^{-2}
4.0	5.62×10^{-3}	7.01×10^{-3}	8.40×10^{-3}	9.71×10^{-3}	1.12×10^{-2}
4.5	4.96×10^{-3}	6.21×10^{-3}	7.46×10^{-3}	8.64×10^{-3}	1.00×10^{-2}
5.0	4.38×10^{-3}	5.57×10^{-3}	6.76×10^{-3}	7.92×10^{-3}	9.22×10^{-3}
5.5	3.95×10^{-3}	5.13×10^{-3}	6.33×10^{-3}	7.48×10^{-3}	8.77×10^{-3}
6.0	3.65×10^{-3}	4.83×10^{-3}	6.03×10^{-3}	7.17×10^{-3}	8.45×10^{-3}
6.5	3.41×10^{-3}	4.57×10^{-3}	5.76×10^{-3}	6.88×10^{-3}	8.15×10^{-3}
7.0	3.18×10^{-3}	4.30×10^{-3}	5.46×10^{-3}	6.54×10^{-3}	7.80×10^{-3}
7.5	2.91×10^{-3}	3.98×10^{-3}	5.07×10^{-3}	6.10×10^{-3}	7.32×10^{-3}
8.0	2.59×10^{-3}	3.55×10^{-3}	4.56×10^{-3}	5.52×10^{-3}	6.67×10^{-3}
8.5	2.16×10^{-3}	2.99×10^{-3}	3.86×10^{-3}	4.69×10^{-3}	5.72×10^{-3}
9.0	1.51×10^{-3}	2.19×10^{-3}	2.85×10^{-3}	3.44×10^{-3}	4.21×10^{-3}

that no titrations gave points which deviated systematically from linearity and therefore none were rejected. The derivatives (dC_H/dC_L) and (dC_H/dC_M) are shown in Table 51. The free ligand and free metal concentrations, shown in Table 52, were calculated from the (dC_H/dC_L) and (dC_H/dC_M) data and $p[L]_o$ and $p[M]_o$ chosen by methods described previously. The constants of complexes forming between lead and glutathione are shown in Table 53.

The quantities ΔC_H, ΔC_L and ΔC_M, shown on Figure 28, were used in the deduction of a model for the lead-glutathione interaction. As was observed in the cadmium system, ΔC_H is displaced to slightly smaller values by about 1×10^{-4} moles/kg. The curves are almost identical to those of the cadmium system. The only observable difference is that the concentration of the complex M(HL) is expected to be slightly higher since the values of ΔC_H, ΔC_L and ΔC_M are larger in magnitude within the pH range 4 to 5. The deprotonation of the (HL) unit occurs in exactly the same pH region as for the cadmium system indicating that loss of that proton is not occurring by displacement by the metal ion.

The middle titration was simulated using the constants shown in Table 53 and agrees very well with the experimental data. This indicates that the model for metal-ligand interaction is complete and reliable.

Table 51. The Derivatives $(dC_H/dC_L)_{C_M,pH}$ and $(dC_H/dC_M)_{C_L,pH}$ for the Lead-Glutathione Experiments.

pH	$(dC_H/dC_L)_{C_M,pH}$	$(dC_H/dC_M)_{C_L,pH}$
3.5	2.488	-0.093
4.0	2.194	-0.199
4.5	1.992	-0.557
5.0	1.922	-0.902
5.5	1.916	-1.118
6.0	1.910	-1.302
6.5	1.888	-1.458
7.0	1.842	-1.593
7.5	1.757	-1.693
8.0	1.634	-1.713
8.5	1.432	-1.530
9.0	1.087	-1.073

Table 52. The Free Ligand and Free Metal Concentrations for the Lead-Glutathione Experiments with $p[L]_o = 12.752$ and $p[M]_o = 3.140$ at pH_o 4.0

pH	Glutathione [L], moles/kg	Lead [M], moles/kg
4.0	1.77×10^{-13}	7.24×10^{-4}
4.5	1.95×10^{-12}	4.80×10^{-4}
5.0	1.84×10^{-11}	2.04×10^{-4}
5.5	1.67×10^{-10}	6.33×10^{-5}
6.0	1.51×10^{-9}	1.57×10^{-5}
6.5	1.35×10^{-8}	3.20×10^{-6}
7.0	1.16×10^{-7}	5.50×10^{-7}
7.5	9.23×10^{-7}	8.26×10^{-8}
8.0	6.53×10^{-6}	1.15×10^{-8}
8.5	3.85×10^{-5}	1.73×10^{-9}
9.0	1.66×10^{-4}	3.80×10^{-10}

Table 53. The Formation Constants of Complexes of Lead and Glutathione.[a]

Species	Formation Constant, β	Log(β)
M(HL)	1.74×10^{16}	16.24
$M(HL)_2$	8.58×10^{30}	30.93
$M(HL)M(HL)_2$	1.67×10^{50}	50.22
M(HL)(L)	3.49×10^{21}	21.54
$M(HL)_3$	1.36×10^{44}	44.13
$M(HL)_2(L)$	3.17×10^{35}	35.50

[a] The standard deviation of the fit to the metal and ligand mass balances are 2.60% and 0.71% respectively. The protonation constants for H_3L and H_2L were determined to be 4.47×10^{21} and 1.59×10^{18} respectively; the protonation constant for HL was given as 3.17×10^{9}.

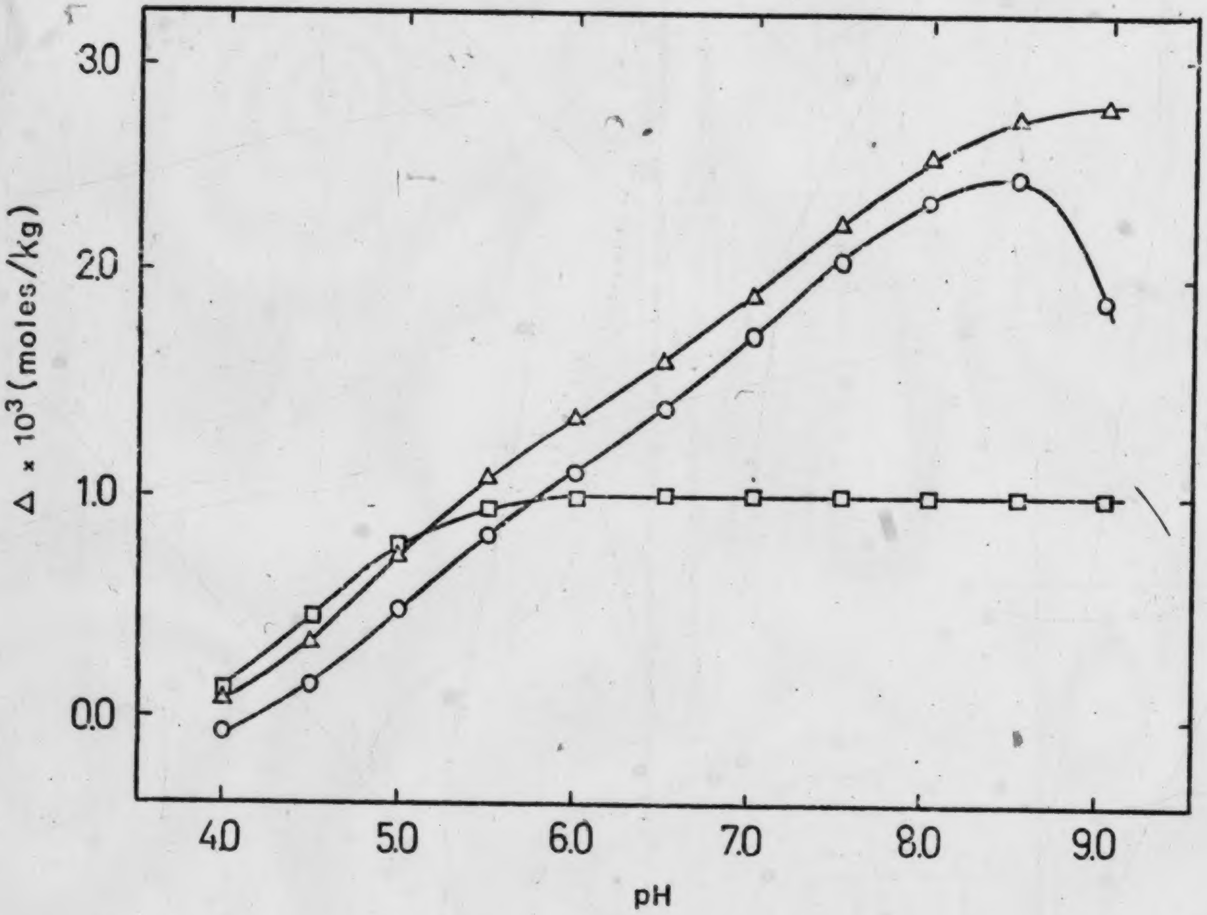


Figure 28. ΔC_H, o; ΔC_L, ▵ ; and ΔC_M, ▫ ; for the Lead-Glutathione system.

C. Discussion.

The complexation of zinc, cadmium and lead by glutathione has been studied by several groups. Martin and Edsall (8) reported formation constants of complexes of zinc, Perrin and Watt (6) discussed zinc and cadmium, Fuhr and Rabenstein (7) and Williams (5) considered zinc, cadmium and lead. Fuhr and Rabenstein employed NMR techniques to study the proton and ^{13}C chemical shifts for nuclei in various parts of the glutathione molecule while the other groups used pH titration methods. Perrin and Watt, and Williams used the program SCOGS to evaluate the pH titration data.

The results presented by the groups using SCOGS disagree with the NMR results. More specifically, both groups concluded that species existed that were impossible according to the chemical shifts observed in the NMR spectra of glutathione. It should be noted here that the papers were published in the order Perrin and Watt, Fuhr and Rabenstein, and Williams. The last group had benefit of the guidance offered by the NMR results in the selection of models of complexation, but chose models virtually identical to those of Perrin and Watt.

The results for the complexation of zinc, cadmium and lead presented in this thesis agree with the chemical shift data presented by Fuhr and Rabenstein. In addition, where possible, titrations were simulated on the basis of models presented by Perrin and Watt and by Williams. These have been compared to data collected for use with the FICS method and to curves simulated on the basis of the models presented in this thesis. These titrations agree well with each other.

Zinc-Glutathione

The model of the interactions betwee zinc and glutathione proposed by Perrin and Watt and by Williams included the major complexes M(HL), M(L), M$(HL)_2$ and M(HL)(L). The NMR data of Fuhr and Rabenstein suggested that no complex M(L) formed in the pH range suggested by those workers. The complex M(L) may, or may not, be real. Molecular models show that it is possible because the sulfhydryl group and the glutamyl amino and carboxyl groups may bind simultaneously to a metal, leaving the ligand in a completely deprotonated form. Two arrangements are possible, one in which all the coordinating groups are planar and a second in which they are immediately adjacent to each other. With this second form, two ligands may bind simultaneously as M$(L)_2$. Fuhr and Rabenstein offered two criticisms of this model. They argued that there is no reason to believe that the metal has sufficient affinity for the ligand to displace the amino proton of the glutamyl residue and, that even if such affinity existed, a large and unstable ten-membered ring would form. Molecular models show, however, that the carboxyl and amino groups of the glutamyl peptide linkage are very close to the metal ion, though not directed toward it and may contribute to the strength of binding of the terminal glutamyl residues and stabilize the large ring. However, the NMR studies of the interaction of zinc and glutathione clearly show that no binding to the glutamyl terminal occurs before pH 6. Both Perrin and Watt, and Williams suggest about 10% of the metal bound in a complex such as M(L) at pH 6. At pH 7 Perrin and Watt suggest that 55% of the metal exists as M(L), the NMR results show about 25%. A species distribution for the constants derived from the FICS method (Table 41) is shown on Figure 24. No species M(L) was found to exist. Deprotonation of the amino group of the glutamyl residue and complexation to the metal begins at pH 6 when $M(HL)_2$ loses a proton to form M(HL)(L). There may be in fact two forms of complexes with that stoichiometry, namely a binding and nonbinding form. However, judging by the pH range of deprotonation it appears to be a displacement type reaction. At pH 7 about 30% of the metal is complexed in a form M(HL)(L).

Martin and Edsall have suggested the three complexes M(HL), $M(HL)_2$ and M(HL)(L), reporting a log(K) for the reaction M + HL = M(HL) of 5.10 and a pK for the first ionization of $M(HL)_2$ of 7.50. Perrin and Watt found 4.74 and 7.04 for these reactions; Williams obtained the values 4.88 and 7.35. From the data in Table 41 values of 5.00 and 7.15 can be calculated for these equilibria. This is

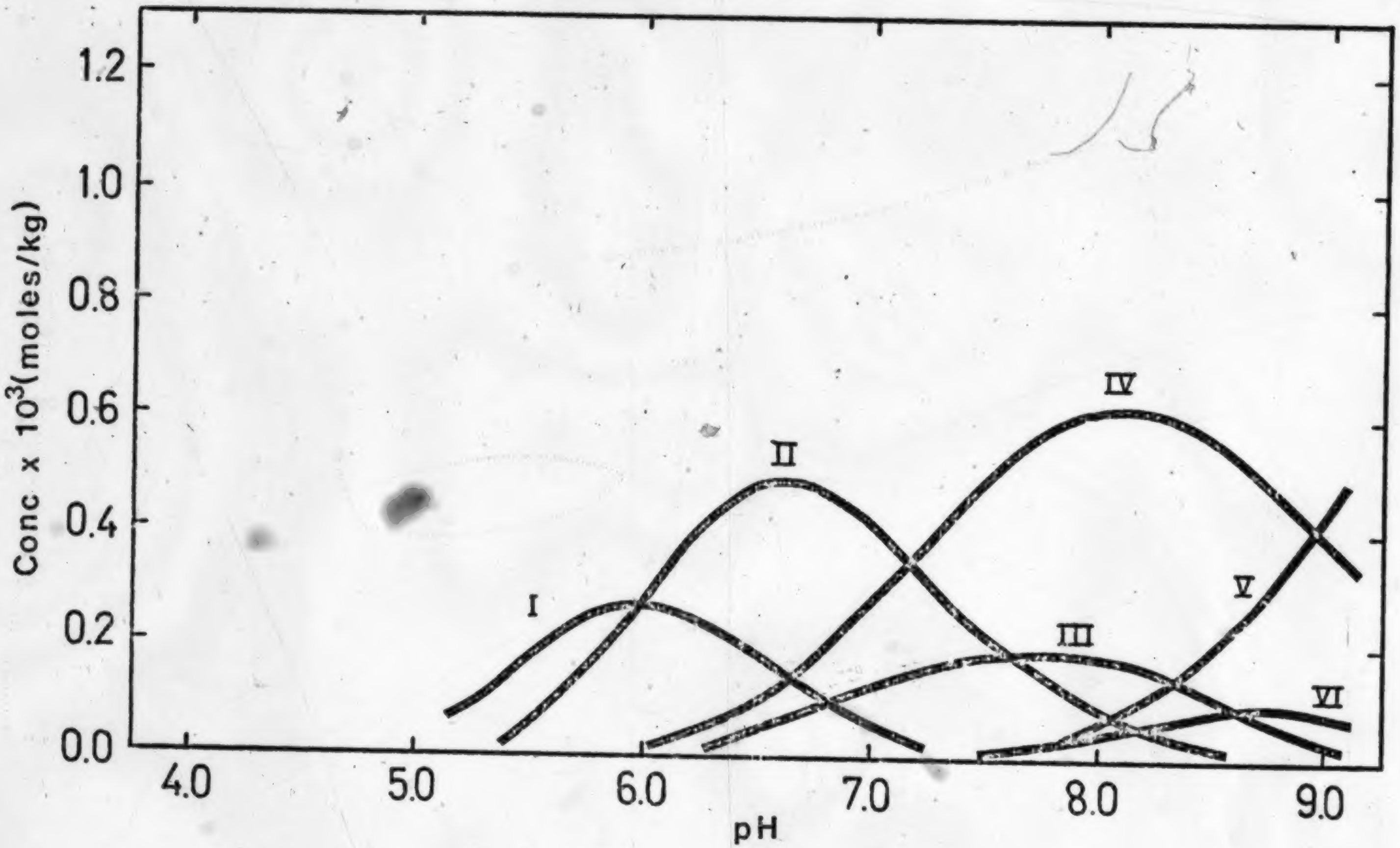


Figure 29. Species distribution for Zinc-Glutathione; M(HL), I; $M(HL)_2$, II; $M(HL)_3$, III; M(HL)(L), IV; $M(L)_2$, V; $M(HL)_2(L)$, VI.

a reasonable situation since Perrin and Watt, and Williams, by including M(L), have probably underestimated the constant for M(HL) and, Martin and Edsall, without adequate knowledge of the complexation above pH 7.0, have overestimated the pK_a of $M(HL)_2$.

Previous studies have not take into account that complexes with a ligand to metal ratio greater than two might exist, even though experiments at glutathione to metal ratios up to 8:1 have been used. The complex $M(HL)_3$ was found to be present using the FICS method, accounting for 20% of the zinc complexed to glutathione at pH 7.8. Molecular models show that three ligands may bind to the metal through the sulfhyrdryl group with no steric interactions at the other parts of the molecules. The net charge on such a complex is -4. However, since the entire complex is quite large and at least 3 of the charges are located on the glycine carboxyl groups at the remote extremities of the complex, this is not an unreasonably large net charge. No evidence was found for a species $M(HL)_4$, which would have a -6 charge.

The models for the interactions of zinc with glutathione suggested by those working with SCOGS and this work with FICS appear quite different and it was of interest to compare titration curves simulated using the alternative models. The curves were found to agree quite well. This is reasonable since it is conceivable that different models may give rise to the same titration curves. This problem has in part been overcome by the use of the [L] and [M] provided by the FICS method, which considerably restricts possible models to explain the data. Attempts to fit the models of Perrin and Watt, and Williams with the [L] and [M] [M] data from the zinc-glutathione experiments failed.

Cadmium-Glutathione

The interactions between cadmium and glutathione appear to be quite different from those involving zinc. Complexation begins at much lower pH and appears to strongly favour complexes bound only through the sulfur of the cysteinyl residue. Perrin and Watt suggest that the major complexes are M(HL), $M(HL)_2$, M(HL)(L), M_2L and ML. They found that at pH 5.8 about 20% of the metal is bound as M_2L. Williams concluded that the major species are M(HL), $M(HL)_2$, M(HL)(L) and M(L). Their results differed from those of Perrin and Watt in that they found about 50% of the cadmium held as M(HL) at pH 4.0 compared to less than 5% for the other workers and they found no M_2L. The discrepancy here indicates that the model of complexation may be rather difficult to establish with potentiometric data alone. The NMR results of Fuhr and Rabenstein indicated that both of the above studies were in some aspects incorrect. A model for the cadmium-glutathione system must explain: (1) the binding to sulfhydryl at pH > 2.0, (2) binding to glutamyl terminal above pH 6.5 and, (3) binding to glycyl terminal in the pH range 2 -10. The results of the FICS method are able in part to explain these observations; however, the model remains incomplete.

The major species found included M(HL), $M(HL)_2$, $M(HL)_3$ and $M(HL)_2(L)$. A species distribution is shown on Figure 30. All of these complexes are probably bound exclusively through the sulfhydryl group and the deprotonation of $M(HL)_3$ occurs without interaction with the metal ion (the pK_a for this ionization was 8.46). In addition to these complexes, a polynuclear species $M(HL)M(HL)_2$ was found, accounting for about 40% of the metal at pH 5.6. It is most interesting to note the similarity of this complex with M_2L found by Perrin and Watt; first, the maximum in concentrations occurred at the same pH; and second, both accounted for 40% of the complexed metal at that pH. The formation of M_2L from H_2L would release 2 protons, while the formation of $M(HL)M(HL)_2$ would release 3. Perrin and Watt's model compensated for this discrepancy in the number of protons titrated to pH 5.7 by including M(L) at that pH.

The polynuclear complex $M(HL)M(HL)_2$ is able to explain the observed binding of the metal ions to the glycine carboxyl group. The glycine group would be able to form bridges to the metal ions of less than fully coordinated complexes thus forming a bonding between $M(HL)_2$ and the singly coordinated M(HL). This process does not itself release protons but it is observed by the effect it will have upon other equilibria involving proton displacements.

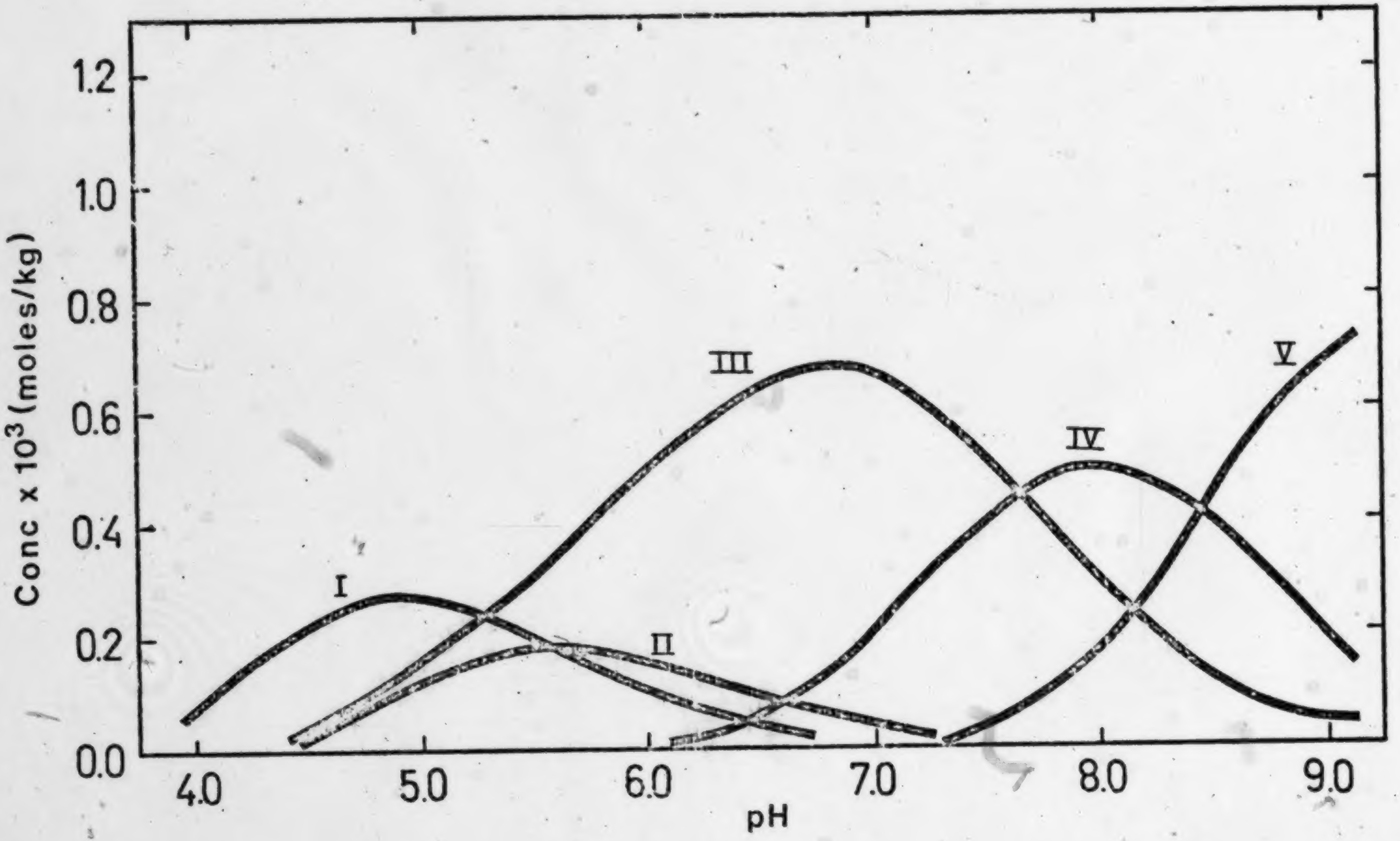


Figure 30. Species distribution for Cadmium-Glutathione; $M(HL)$, I; $M(HL)M(HL)_2$, II; $M(HL)_2$, III; $M(HL)_3$, IV; $M(HL)_2(L)$, V.

The model suggested by the FICS method does not account for the observed binding of cadmium to the glutamyl amino group. The species distribution shown on Figure 30 indicates that a deprotonation of $M(HL)_3$ occurs at pH 7, but this is probably not a displacement by the metal ion. The inadequacy of the model is further shown by a difference between the actual titration curves and simulated data in the pH range 6.5 to 8.5. This strongly suggests that a displacement reaction does occur in that pH range, with binding to the amino group of the glutamyl residue. The models used by the other workers do not offer much help since they propose formation of M(L) as low as pH 5.0 and M(HL)(L) at pH 6.0. With the data from the FICS method a model including M(HL)(L) indicates that it would only begin to form above pH 7.0 and would not offer explanation of the NMR results. The reason for these problems may stem from a polymerization reaction where the uncomplexed glutamyl and glycyl terminals may bind to metals other than that to which the sulfhydryl group is bound. This type of situation has been reported for glutathione complexes of nickel (54, 55) and silver (74). In the latter report, silver-glutathione polymers were separated and characterized, and found to have between 15 and 20 metal-glutathione units. If series of short polymers are present in cadmium-glutathione solutions this would pose considerable difficulty for pH titration methods; first, a variety of complexes might exist in a narrow pH range; and second, the mixture may not be at equilibrium. In either case, potentiometric titrations will fail to resolve the situation.

In spite of these difficulties a great deal of information about the complexation of cadmium to glutathione has been extracted from the titration data. First, it should be remembered that the deduction of a model of complexes existing in solution and formation constants is only a part of the FICS method. The values for [L] and [M] shown in Table 46, the values of the derivatives in Table 45 and the delta quantities of Figure 27 have been derived from the experimental data and are independent of a model for complexation. They will not be affected by the choice of an alternative model.

Below pH 7.0 the majority of metal is held in the complexes M(HL), $M(HL)M(HL)_2$ and $M(HL)_2$. These species account for both the observed pH titration curves and the NMR data. Models proposed by Perrin and Watt, and by Williams did not agree with the NMR observations. This model also describes the complexes which may form in physiological pH ranges. In this respect it is interesting to note that glutathione in physiological media will invariably be bound to heavy metals only at the sulfhydryl group. The other parts of the molecule will continue to behave as if the metal was not present, perhaps binding to other metal ions, to enzymes and to proteins.

Lead-Glutathione

The reaction of lead with glutathione has been found to be almost identical to that of cadmium. The curves of ΔC_H, ΔC_L and ΔC_M shown in Figure 23, and the species distribution shown in Figure 31,are very similar to those for the cadmium system. The NMR data collected by Fuhr and Rabenstein showed that the interaction of lead with glutathione occurs through the sulfhydryl and glycine carboxyl groups in essentially the same way as in the cadmium system. However, there was a major difference; no complexation of lead to the glutamyl terminal was observed below pH 12.0. This suggests that the polymerization reactions of the type proposed above for cadmium are not likely in the lead system. The actual titration data was matched very well by a simulated titration curve based on the model including the species M(HL), $M(HL)M(HL)_2$, $M(HL)_2$, $M(HL)_3$ and $M(HL)_2(L)$.

The model for the complexation of lead by glutathione described by Williams included the complexes ML, M(HL)(L) and ML_2. All three of these species form by loss of an amino proton from the glutamyl group below pH 8.0. Since the amino proton would usually be titrated above pH 9.0, these reactions must occur through displacement of the protons by metal ions. This does not agree with NMR data, which shows that the amino group does not participate in binding below pH 12.0. Williams made no attempt to use

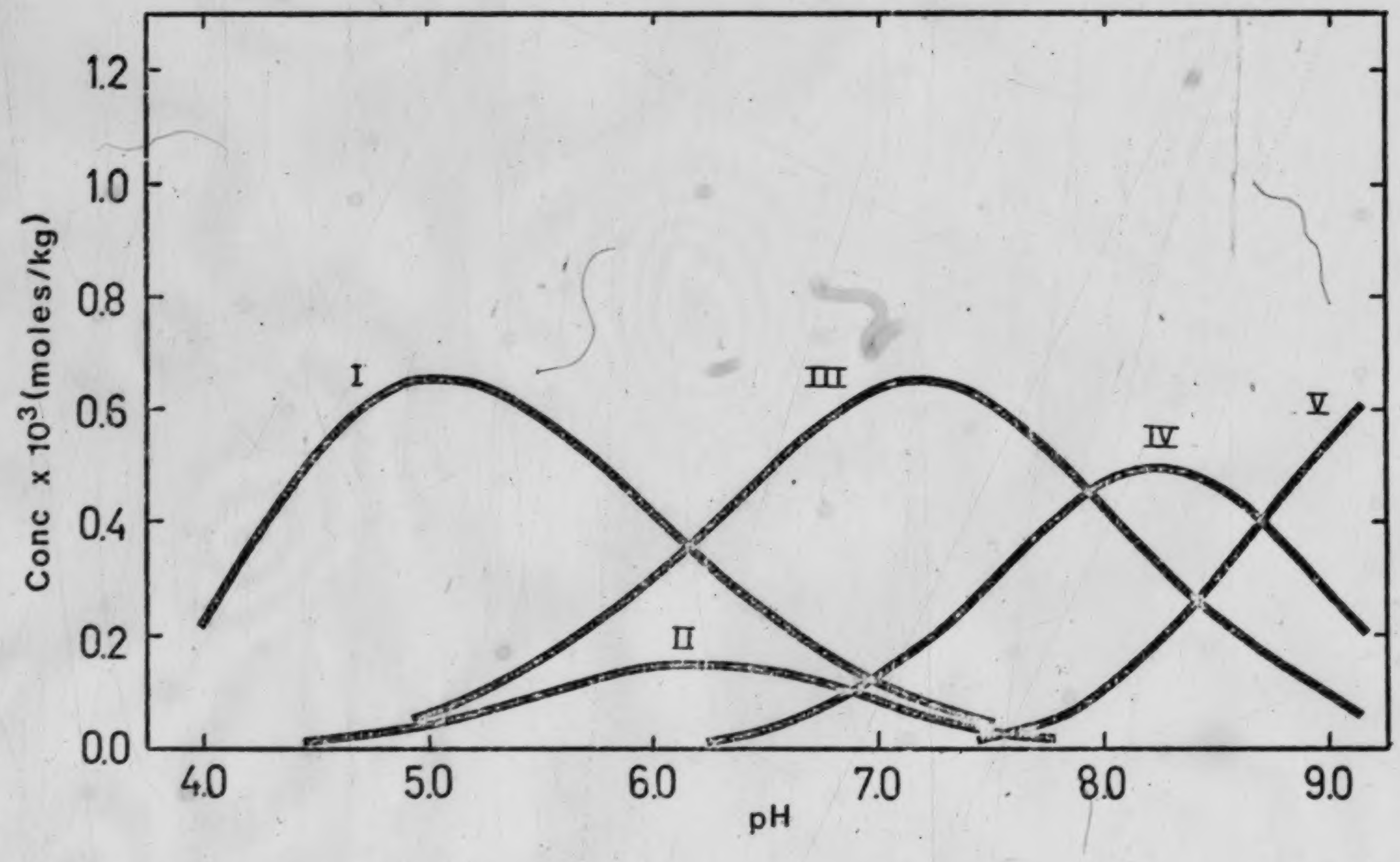


Figure 31. Species Distribution for Lead-Glutathione; M(HL), I; M(HL)$M(HL)_2$, II; $M(HL)_2$, III; $M(HL)_3$, IV; $M(HL)_2$(L), V.

the NMR data as a guide to selection of a model of complexation.

As was described in the section on the binding of cadmium, some possible reactions of glutathione with heavy metals in physiological media are suggested by the results of the experiments in this thesis. It appears that only very weak complexation occurs to groups other than the sulfhydryl within physiological pH ranges and, if binding does occur, it is most likely to another metal ion through the glycine terminal. This indicates that the parts of glutathione molecule that are not directly involved with binding behave as if the binding had not occurred. This has implications in the understanding of the effect of heavy metals on biochemical processes. For example, should a lead-glutathione complex bind to an enzyme through the glutamyl terminal, the activity of that enzyme may be effectively quenched. In this way metal ions which would not normally interact with enzymes near their active sites may, through a carrier molecule, have an effect on the activity of that enzyme. This also suggests that glutathione cannot be used as a heavy metal sequestering agent. Aside from the problems involving the other chemical properties of glutathione it cannot be used as a drug for treatment of metal poisoning because, as described above, it retains a great deal of its previous character even after complexation. As a result it will continue to participate in biochemical processes, perhaps spreading the metal faster. This reduces the number of routes by which the metal ions might be eliminated from the body.

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APPENDIX A

THE COMPUTER PROGRAM USED TO SIMULATE TITRATION CURVES.

```
5 DIM F$(20),G$(20),H(10),L(10),M(10),B(10),M1(10),P(20),
                                    L1(10),P1(10),P2(10)
10 PRINT "NUMBER OF TITRATIONS?"\INPUT S5
20 FOR I=1 TO S5
30 PRINT "CONC LIGAND,METAL"\INPUT S6(I),S7(I)
40 PRINT "FILE FOR STORAGE?"\INPUT F$(I)
50 NEXT I
80 PRINT "INITIAL VOL?"\INPUT V1
90 PRINT "NUMBER OF ITERATIONS?"\INPUT Q1
100 PRINT "INIT PH,NUMBER OF PH,PH INCR."\INPUT P2,N,I1
110 PRINT "NUMBER OF PROTONS,BETA FOR MOST PROTONATED FORM?"
                                                  \INPUT H,B
120 PRINT "NUMBER OF L INCREMENTS,RATIO,RATL??"\INPUT N3,R1,R5
130 PRINT "NUMBER OF M INCR,RATIO,RATM??"\INPUT M3,R6,R7
140 PRINT "NUMBER OF SPECIES?"\INPUT N1
150 FOR I=1 TO N1
160 PRINT "NUMBER OF H,L,M,AND BETA?"\INPUT H(I),L(I),M(I),B(I)
170 NEXT I
210 PRINT "ADDED ACID?"\INPUT C1
220 PRINT "CONC OF NAOH?"\INPUT B5
230 FOR S4=1 TO S5
231 LET H$=F$(S4)
235 OPEN H$ FOR OUTPUT AS FILE #5
245 LET L=S6(S4)\LET M=S7(S4)\LET P=P2
250 LET X1=P-I1\LET X1=EXP(-X1*LOG(10))
260 LET X2=EXP(-P*LOG(10))
270 LET C=C1+H*L
280 PRINT "CHO EQUALS",C
300 LET F1=(EXP(H*LOG(X1))-EXP(H*LOG(X2)))/EXP(H*LOG(X2))
305 LET F2=0
310 LET L1=L/(B*EXP(H*LOG(X1)))
315 LET M4=M
325 LET W=1.00000E-14
330 LET L2=L\LET M2=M
350 LET V=0
360 FOR I=1 TO N
370 LET P1=EXP(-P*LOG(10))
380 LET L3=L1\LET M5=M4
390 LET L1=L1*(1+F1)\LET R=L1*(F1+.8)/R1
400 IF F1>=0 THEN 410 \LET R=L1*(F1-.8)/R1
410 LET I2=R/N3\LET I3=I2\LET L1=L1-(R/2)
420 FOR Q=1 TO N3
430 LET L1(Q)=L1\LET L1=L1+I2\NEXT Q
440 LET M4=M4*(1+F2)\LET R=M4*(F2+.8)/R6
450 IF F2>=0 THEN 460 \LET R=M4*(F2-.8)/R6
460 LET I2=R/M3\LET I4=I2
465 LET M4=M4-(R/2)
470 FOR Q=1 TO M3
480 LET M1(Q)=M4\LET M4=M4+I2\NEXT Q
```

```
600 FOR Q=1 TO N1
610 LET P(Q)=H(Q)*LOG(P1)\NEXT Q
611 FOR Q=1 TO Q1
612 LET Y=1
613 IF Q=1GO TO 750
616 LET I3=I3*1.0001\LET I4=I4*1.001
617 LET Y=1
630 LET I2=I3*R5/(EXP((Q-1)*LOG(N3))+R5)
640 LET L1=F5-.5*N3*I2
650 FOR J=1 TO N3
660 LET L1(J)=L1\LET L1=L1+I2\NEXT J
670 LET I2=I4*R7/(EXP((Q-1)*LOG(M3))+R7)
680 LET M4=F6-.5*M3*I2
690 FOR J=1 TO M3
700 LET M1(J)=M4\LET M4=M4+I2\NEXT J
750 FOR K=1 TO N3
760 FOR K1=1 TO N1
770 LET P1(K1)=L(K1)*LOG(L1(K))
771 NEXT K1
780 FOR O=1 TO M3
790 FOR O1=1 TO N1
800 LET P2(O1)=M(O1)*LOG(M1(O))
801 NEXT O1
810 FOR K1=1 TO N1
820 LET S8=LOG(B(K1))+P(K1)+P1(K1)+P2(K1)
825 IF S8>=-35 THEN 826 \LET X=0
826 IF S8<-35 THEN 830 \LET X=EXP(S8)
830 LET S1=S1+H(K1)*X
840 LET S2=S2+L(K1)*X
850 LET S3=S3+M(K1)*X\NEXT K1
860 LET S1=P1-W/P1+S1
870 LET S2=L1(K)+S2
880 LET S3=M1(O)+S3
900 LET Z=Y
910 LET Y=ABS(L-S2)+ABS(M-S3)
930 IF Y-Z<=0GO TO 950
940 LET Y=Z\GO TO 1000
950 LET F5=L1(K)\LET F6=M1(O)
960 LET A3=S1\LET A4=S2\LET A5=S3
970 LET C2=O\LET C3=K
1000 LET S1=0\LET S2=0\LET S3=0
1010 NEXT O
1020 NEXT K
1040 IF C2=1GO TO 616
1050 IF C2=M3GO TO 616
1060 IF C3=1GO TO 616
1065 IF C3=N3GO TO 616
1070 FOR K=1 TO 5
1080 LET V=(C*V1)/(V1+V)-A3
1090 LET V=(V*V1)/(B5-V)
1095 IF V>=0 THEN 1100 \LET V=0
1100 NEXT K
```

```
1110 LET L=L2*V1/(V+V1)
1115 LET M=M2*V1/(V+V1)
1120 NEXT Q
1125 PRINT Y,F5,F6,C2,C3
1130 LET L1=F5\LET M4=F6
1140 LET F1=(L1-L3)/L3
1145 LET F2=(M4-M5)/M5
1150 PRINT P,V,A3,A4,A5
1151 PRINT #5:P,",",A3,",",V
1160 LET P=P+I1
1170 NEXT I
1180 CLOSE #5
1200 NEXT S4

READY
```

APPENDIX B

A SIMPLE DERIVATION OF THE BASIC RELATIONSHIPS IN THE FICS METHOD

$$C_L = [L] + [HL] + [HML_2] \qquad (1)$$

$$= [L] + \beta_{HL}[H][L] + 2\beta_{HML_2}[H][M][L]^2 \qquad (2)$$

$$C_M = [M] + \beta_{HML_2}[H][M][L]^2 \qquad (3)$$

$$C_H = [H^+] - [OH^-] + \beta_{HL}[H][L] + \beta_{HML_2}[H][M][L]^2 \qquad (4)$$

$$\frac{dC_L}{d[H]} = \beta_{HL}[L] + 2\beta_{HML_2}[M][L]^2 \qquad (5)$$

$$\frac{dC_M}{d[H]} = \beta_{HML_2}[M][L]^2 \qquad (6)$$

$$\frac{dC_H}{d[L]} = \beta_{HL}[H] + 2\beta_{HML_2}[H][M][L] \qquad (7)$$

$$\frac{dC_H}{d[M]} = \beta_{HML_2}[H][L]^2 \qquad (8)$$

From Equations 5 and 7:

$$[H]\left(\frac{dC_L}{d[H]}\right) = [L]\left(\frac{dC_L}{d[L]}\right) \qquad (9)$$

Since $\frac{d[H]}{[H]}$ is equal to dln[H],

$$\frac{dC_L}{d\ln[H]} = \frac{dC_H}{d\ln[L]} \qquad (10)$$

$$\frac{d\ln[L]}{d\ln[H]} = \frac{dC_H}{dC_L} \qquad (11)$$

Since -log[H] = p[H], and -log[L] = p[L],

$$\frac{dp[L]}{dp[H]} = \frac{dC_H}{dC_L} \qquad (12)$$

√

Integration of both sides from pH_o to pH gives:

$$\int_{pH_o}^{pH} \left(\frac{dp[L]}{dpH}\right) dpH = \int_{pH_o}^{pH} \left(\frac{dC_H}{dC_L}\right) dpH \qquad (13)$$

$$\int_{pH_o}^{pH} dp[L] = \int_{pH_o}^{pH} \left(\frac{dC_H}{dC_L}\right) dpH \qquad (14)$$

$$p[L]\Big|_{pH_o}^{pH} = \int_{pH_o}^{pH} \left(\frac{dC_H}{dC_L}\right) dpH \qquad (15)$$

And if p[L] at pH_o is equal to $p[L]_o$:

$$p[L] = p[L]_o - \int_{pH_o}^{pH} \left(\frac{dC_H}{dC_L} \right) dpH \tag{16}$$

Equations 6 and 8 can be combined:

$$[H]\left(\frac{dC_M}{d[H]} \right) = [M]\left(\frac{dC_H}{d[M]} \right) \tag{17}$$

This equation can be manipulated in exactly the same manner as Equation 9 and will produce:

$$p[M] = p[M]_o - \int_{pH_o}^{pH} \left(\frac{dC_H}{dC_M} \right) dpH \tag{18}$$

Equations 16 and 18 are Equations 9 and 10 in Chapter II.

APPENDIX C

THE COMPUTER PROGRAM USED TO STANDARDIZE pH ELECTRODES

```
BUFFER      BASIC V01B-02

100 DIM C(10),R(10)
150 PRINT "HOW MANY BUFFERS DO YOU HAVE? >3AND<6"\INPUT N1
170 FOR J=1 TO N1
180 PRINT "** BUFFER,PH? **"\INPUT C(J)
300 PRINT "BUFFER,MV?"\INPUT A1
405 LET R(J)=A1
406 NEXT J
420 LET S1=0\LET S2=0\LET U=0\LET W=0\LET P=0
460 FOR I=1 TO N1
470 LET S1=S1+R(I)
480 LET S2=S2+C(I)
490 LET U=U+R(I)*R(I)
500 LET W=W+C(I)*C(I)
510 LET P=P+R(I)*C(I)
511 NEXT I
520 LET U1=N1*U-S1*S1
530 LET W1=N1*W-S2*S2
540 LET P1=N1*P-S1*S2
550 LET S3=P1/U1
560 LET S4=(S2-S3*S1)/N1
570 LET D2=0
580 FOR I=1 TO N1
590 LET D1=C(I)-(S3*R(I)+S4)
600 LET D2=D2+D1*D1
601 NEXT I
610 LET V=D2/(N1-2)
620 LET V1=SQR((N1*V)/U1)
630 LET V2=SQR((U*V)/U1)
640 PRINT "SLOPE",S3\PRINT "INTERCEPT",S4
660 PRINT "STANDARD DEVIATION IN THE SLOPE",V1
670 PRINT "STANDARD DEVIATION IN THE INTERCEPT",V2
```

APPENDIX D

THE COMPUTER PROGRAM TTR1 WHICH CONTROLLED THE COLLECTION OF EXPERIMENTAL DATA

```
      REAL MINMV,MAXMV,MAXSD,MAXSLO,MV
      INTEGER P1,P2
      REAL LLEAST
      DIMENSION C(150),D(150),WTB1(150),WTB2(150),PPH(150)
      DIMENSION A(50),B(50)
      COMMON CB1,CB2,C1,C2
6     FORMAT(1PE12.5)
      CALL IDOR(0,0,"4,"4)
7     FORMAT(' ','AUTO=0,MANUAL=1')
      WRITE(7,7)
      ACCEPT 9,I
      IF(I) 100,100,50
100   CONTINUE
      DATA E1,E2,IDOWN/0.03,0.0,450/
      DATA MINMV,MAXMV,DANMV/5.0,10.0,15.0/
      DATA MINDOW,MAXDOW,N/450,3000,10/
      DATA MAXSD,MAXSLO,ENDMV/0.1,1.0,400.0/
      DATA NEB,CB1,CB2/0,1.0E-2,1.0E-2/
      DATA C1,C2/1.0E-3,2.0E-3/
      GO TO 60
50    CONTINUE
8     FORMAT(' ',3F10.4)
9     FORMAT(' ',5I5)
10    FORMAT(' ','INFORMATION FROM CALIBRATION, SLOPE?')
      WRITE(7,10)
      ACCEPT 8,E1
11    FORMAT(' ','INTERCEPT?')
      WRITE(7,11)
      ACCEPT 8,E2
12    FORMAT(' ','DURATION OF FIRST DELIVERY?')
      WRITE(7,12)
      ACCEPT 9,IDOWN
13    FORMAT(' ','MINIMUM CHANGE EXPECTED?')
      WRITE(7,13)
      ACCEPT 8,MINMV
14    FORMAT(' ','MAXIMUM CHANGE EXPECTED?')
      WRITE(7,14)
      ACCEPT 8,MAXMV
15    FORMAT(' ','A DANGEROUS CHANGE WOULD BE?')
      WRITE(7,15)
      ACCEPT 8,DANMV
16    FORMAT(' ','THE MINIMUM DELIVERY TIME?')
      WRITE(7,16)
      ACCEPT 9,MINDOW
```

```
17      FORMAT(' ','THE MAXIMUM DELIVEY TIME?')
        WRITE(7,17)
        ACCEPT 9,MAXDOW
18      FORMAT(' ','NUMBER OF POINTS TO BE SAMPLED?')
        WRITE(7,18)
        ACCEPT 9,N
19      FORMAT(' ','MAXIMUM ALLOWED STANDARD DEVIATION?')
        WRITE(7,19)
        ACCEPT 8,MAXSD
20      FORMAT(' ','MAXIMUM SLOPE ALLOWED?')
        WRITE(7,20)
        ACCEPT 8,MAXSLO
21      FORMAT(' ',7X,'SD',10X,'SLOPE',15X,'WEIGHT',10X,
22      FORMAT('0')                          'MV',15X,'PH')
23      FORMAT(' ','END OF TITRATION?,MV')
        WRITE(7,23)
        ACCEPT 8,ENDMV
26      FORMAT(' ','NUMBER OF EXTRA BURETS?')
        WRITE(7,26)
        ACCEPT 9,NEB
27      FORMAT(' ','CONC IN NUMBER 1?')
        WRITE(7,27)
        ACCEPT 8,CB1
28      FORMAT(' ','CONC IN NUMBER 2?')
        WRITE(7,28)
        ACCEPT 8,CB2
29      FORMAT(' ','CONC OF COMPONANT 1 IN TITR CELL?')
        WRITE(7,29)
        ACCEPT 8,C1
31      FORMAT(' ','CONC OF COMPONANT 2?')
        WRITE(7,31)
        ACCEPT 8,C2
41      FORMAT(' ','RATE OF DRIFT,MG PER 15 MIN')
        WRITE(7,41)
        ACCEPT 8,DRIFT
        DRIFT=DRIFT/9.0E4
60      WRITE(7,35)E1,E2,MINMV,MAXMV,DANMV
        WRITE(7,35)MAXSD,MAXSLO,ENDMV
        WRITE(7,35)CB1,CB2,C1,C2
60      WRITE(7,22)
        WRITE(7,21)
        WRITE(7,22)
        C(1)=0.0
        RUNTIM=0.0
        WTB1(1)=0.0
        WTB2(1)=0.0
        K=0
        OLDMV=0.0
        J=0
```

```
35      FORMAT(5(1PE15.4))
36      FORMAT(' ',1PE12.3,3X,1PE12.3,5X,1PE15.5)
37      FORMAT(' ',1PE12.3,3X,1PE12.3,23X,1PE12.3,5X,1PE12.3)
25      DO 30 I=1,N
        CALL COLLEC(A(I),B(I))
        CALL DELAY(150)
        RUNTIM=RUNTIM+150.0
30      CONTINUE
        J=J+1
        CALL LLEAST(B,WEIGHT,SD,SLOPE,N)
        WRITE(7,36)SD,SLOPE,WEIGHT
        IF (J.GT.1) GO TO 39
        IF(K.EQ.0)OLDWT=WEIGHT
        DELWT=OLDWT-WEIGHT
        CALL VOLUME(NEB,DELWT,P1,P2)
        IF (P1.GT.0) CALL ADELIV(P1)
        RUNTIM=RUNTIM+4*P1
        IF(P2.GT.0) CALL BDELIV(P2)
        RUNTIM=RUNTIM+4*P2
        IF((P1.LT.10).AND.(P2.LT.10)) GO TO 39
        GO TO 25
39      CONTINUE
        CALL LLEAST(A,MV,SD,SLOPE,N)
        PH=E1*MV+E2
        WRITE(7,37)SD,SLOPE,MV,PH
        IF(J.EQ.5) GO TO 40
        IF(ABS(SD).GT.MAXSD) GO TO 25
        IF(ABS(SLOPE).GT.MAXSLO) GO TO 25
40      IF(MV.GT.ENDMV) GO TO 70
        DELMV=ABS(OLDMV-MV)
        IF(DELMV.LT.MINMV) IDOWN=IDOWN*2.0
        IF((DELMV.GT.MINMV).AND.(DELMV.LT.MAXMV)) IDOWN=IDOWN
        IF((DELMV.GT.MAXMV).AND.(DELMV.LT.DANMV)) IDOWN=IDOWN
        IF(DELMV.GT.DANMV) IDOWN=IDOWN/5.0                 /2.0
        IF(IDOWN.LT.MINDOW) IDOWN=MINDOW
        IF(IDOWN.GT.MAXDOW) IDOWN=MAXDOW
        WRITE(7,9)IDOWN
        CALL DELIVE(IDOWN)
        RUNTIM=RUNTIM+30.0+IDOWN
        IF(K.EQ.0) OLDWT=WEIGHT
        K=K+1
        D(K)=MV
        RUNTIM=RUNTIM+500.0
        WRITE(7,8)RUNTIM,DRIFT*RUNTIM
        DELWT=OLDWT-WEIGHT
        DELWT=DELWT-(DRIFT*RUNTIM)/1000.0
        RUNTIM=0.0
        IF(K.GT.1) C(K)=C(K-1)+DELWT
        OLDWT=WEIGHT
        WRITE(7,9)P1,P2
        IF(K.GT.1) WTB1(K)=WTB1(K-1)+(P1*1.0218/10000.0)
        IF(K.GT.1) WTB2(K)=WTB2(K-1)+(P2*1.0218/10000.0)
        PPH(K)=PH
65      CALL DELAY(300)
```

```
      RUNTIM=RUNTIM+300.0
      OLDMV=MV
      J=0
      GO TO 25
70    WRITE(7,35)(C(I),WTB1(I),WTB2(I),D(I),PPH(I),I=1,K)
      WRITE(7,9)K
32    FORMAT(' ','OUTPUT TO FILE FTN1?? YES=0,NO=1')
33    WRITE(7,32)
      ACCEPT 9,I
      GO TO (38,34) I+1
38    WRITE(1,6)(C(J),WTB1(J),WTB2(J),D(J),PPH(J),J=1,K)
      ENDFILE 1
      GO TO 33
34    CONTINUE
      STOP
      END
      SUBROUTINE LLEAST(B,A1,S1,R1,N)
      DIMENSION B(50)
      A1=0.0
      Y2=0.0
      X2=0.0
      X4=0.0
      DO 10 I=1,N
      Z=I
      A1=A1+B(I)
      Y2=Y2+Z*B(I)
      X2=X2+Z
      X4=X4+Z*Z
10    CONTINUE
      Z=N
      Y1=A1
      A1=A1/Z
      D=0.0
      DO 20 I=1,N
      D=D+(B(I)-A1)*(B(I)-A1)
20    CONTINUE
      R1=(Z*Y2-Y1*X2)/(Z*X4-X2*X2)
      S1=SQRT(D/(Z-1.0))
      RETURN
      END
      SUBROUTINE COLLEC(A,B)
      INTEGER TIME
      TIME=1
      CALL IDOR(0,0,"3,"0)
      CALL DELAY(TIME)
      IX=IDIR(0,0,-1,0)
      C=IX
      CALL IDOR(0,0,"3,"1)
      CALL DELAY(TIME)
      IX=IDIR(0,0,-1,0)
      B=IX
      B=(10000.*B+C)/1000.
      CALL IDOR(0,0,"3,"2)
      CALL DELAY(TIME)
      IX=IDIR(0,0,-1,0)
      A=IX
      A=A/10.
      CALL IDOR(0,0,'3,'3)
      RETURN
      END
      SUBROUTINE DELAY(TIME)
      INTEGER TIME
      DO 10 I=1,TIME
      ICMF =0
      CALL SETR(5,0,1.,ICMF)
      CALL LWAIT(ICMF,0)
      CALL SETR(-1,,,)
10    CONTINUE
      RETURN
      END
      SUBROUTINE DELIVE(I)
      CALL IDOR(0,0,'4,'0)
      CALL DELAY(15)
      CALL IDOR(0,0,'4,'4)
      CALL DELAY(I)
      CALL IDOR(0,0,'4,'0)
      CALL DELAY(15)
      CALL IDOR(0,0,'4,'4)
      RETURN
      END
      SUBROUTINE VOLUME(N,V,P1,P2)
      COMMON CB1,CB2,C1,C2
      INTEGER P1,P2
      V1=0.0
      V2=0.0
      GO TO (10,20,30) N+1
10    P1=0
      P2=0
      RETURN
20    V1=(C1*V)/(CB1-C1)
      P1=V1*10000.
      V2=(C2*V)/(CB2-C2)
      P2=V2*10000.
      RETURN
30    DO 40 I=1,5
      V1=(C1*(V+V2))/(CB1-C1)
      V2=(C2*(V+V1))/(CB2-C2)
40    CONTINUE
      P1=V1*10000.
      P2=V2*10000.
      RETURN
      END
      SUBROUTINE ADELIV(N)
      DO 10 I=1,N
      CALL IDOR(0,0,'20,'20)
      CALL DELAY(2)
      CALL IDOR(0,0,'20,'0)
      CALL DELAY(2)
10    CONTINUE
      RETURN
      END
      SUBROUTINE BDELIV(N)
      DO 10 I=1,N
      CALL IDOR(0,0,'40,'40)
      CALL DELAY(2)
      CALL IDOR(0,0,'40,'0)
      CALL DELAY(2)
10    CONTINUE
      RETURN
      END
```

APPENDIX E

THE COMPUTER PROGRAM FCOPY WHICH TRANSLATED DATA COLLECTED IN FORTRAN TO A FORM COMPATIBLE WITH THE LANGUAGE BASIC

```
FCOPY       BASIC V01B-02

40 DIM A(100),B(100),C(100),D(100),E(100)
50 PRINT "FILE FOR INPUT"\INPUT F$
70 OPEN F$ FOR INPUT AS FILE #5
80 I=1
90 INPUT #5:A$,B$,C$,D$,E$
100 A(I)=VAL(A$)
110 B(I)=VAL(B$)
120 C(I)=VAL(C$)
130 D(I)=VAL(D$)
140 E(I)=VAL(E$)
150 IF END #5 THEN 200
160 I=I+1
170 GO TO 90
200 PRINT "***** ",I,"   ***"
205 CLOSE #5
300 PRINT "NUMBER OF COPIES?"\INPUT N
310 FOR J=1 TO N
320 PRINT "FILE FOR OUTPUT?"\INPUT F$
330 OPEN F$ FOR OUTPUT AS FILE #5
340 FOR K=1 TO I
350 PRINT #5:A(K),",",B(K),",",C(K),",",D(K),",",E(K)
360 NEXT K
365 CLOSE #5
370 NEXT J
380 STOP
390 END

READY
```

APPENDIX F

THE COMPUTER PROGRAM WHICH CALCULATED C_H's AT EXACT pH's FROM THE TITRATION DATA OUTPUT FROM PROGRAM TTR1

```
5 DIM P(200),B(200),V(200)
10 PRINT "FILE TO BE USED AS INPUT?"\INPUT F$
20 OPEN F$ FOR INPUT AS FILE #5
25 M=1
26 L=0
30 PRINT "PH INCREMENT?"\INPUT X
40 PRINT "NUMBER OF LIGANDS?"\INPUT N
45 FOR I=1 TO N
50 PRINT "LIGAND CONC.?"\INPUT K
55 PRINT "NUMBER OF PROTONS?"\INPUT K2
60 L=L+K*K2
61 NEXT I
62 PRINT "CONC. OF ADDED ACID?"\INPUT K1
63 PRINT "CONC. OF TITRANT?"\INPUT K3
64 PRINT "TOTAL INIT VOL?"\INPUT K4
70 PRINT "CHO EQUALS",L+K1
71 PRINT "CONC OF H+ IN BURET #1?"\INPUT B5
72 PRINT "CONC OF H+ IN #2?"\INPUT C5
74 REM A=WT OF NAOH,B=VOL FROM BURET #1,C=VOL FROM #2,E=PH
75 INPUT #5:A,B,C,E,E,E
80 LET P=P+X
81 IF P<E THEN 80
100 V2=A\C2=E\R2=B\S2=C
110 IF END #5 THEN 400
120 INPUT #5:A,B,C,E,E,E
200 IF P>E THEN 100
210 V1=A\C1=E\R1=B\S1=C
220 I=((C2*V1)-(C1*V2))/(C2-C1)
230 S=(V1-I)/C1
240 V=S*P+I
245 I=((C2*R1)-(C1*R2))/(C2-C1)
246 S=(R1-I)/C1
247 R3=S*P+I
250 I=((C2*S1)-(C1*S2))/(C2-C1)
251 S=(S1-I)/C1
252 S3=S*P+I
260 B1=(L*K4+R3*B5+S3*C5-K3*V)/(K4+R3+S3+V)
270 P(M)=P\B(M)=B1
275 V(M)=V+R3+S3
280 M=M+1
300 P=P+X
310 GO TO 200
400 PRINT "COPIES OF OUTPUT?"\INPUT N
405 CLOSE #5
410 FOR J=1 TO N
420 PRINT "FILE FOR OUTPUT?"\INPUT F$
430 OPEN F$ FOR OUTPUT AS FILE #5
440 FOR K=1 TO M-1
450 PRINT #5:P(K),",",B(K),",",V(K)
460 NEXT K
465 CLOSE #5
470 NEXT J
```

APPENDIX G

THE COMPUTER PROGRAM WHICH CALCULATED (dC_H/dC_X) AND [L] AND [M] FROM THE C_H OUTPUT OF THE PROGRAM IN APPENDIX F

```
10 DIM R(8),Z(8),V(8),Q(78),C(8,78),D(8,78)
50 PRINT "HOW MANY TITRATIONS?"\INPUT N1
100 PRINT "LOWER LIMIT OG PH?"\INPUT L
150 PRINT "HIGH LIMIT?"\INPUT H
200 FOR I=1 TO N1
250 PRINT "WHICH DATA FILE?"\INPUT F$
260 OPEN F$ FOR INPUT AS FILE #5
300 PRINT "WHAT IS CX THIS TITRATION?"\INPUT Z(I)
320 PRINT "INITIAL VOLUME?"\INPUT V(I)
350 LET J=J+1
400 INPUT #5:A,B,C
420 IF A>=L THEN 450 \LET J=0
450 IF A<=L THEN 451 \IF A>=H THEN 451 \LET C(I,J)=B
                                       \LET Q(J)=A\LET M=J
451 IF A<=L THEN 460 \IF A>=H THEN 460 \LET D(I,J)=C
460 IF END #5 THEN 500
470 GO TO 350
500 PRINT "**END OF FILE**"
550 LET J=0
600 PRINT "NUMBER OF POINTS",M
640 CLOSE #5
650 NEXT I
1000 FOR J=1 TO M
1010 FOR I=1 TO N1
1020 LET R(I)=Z(I)*V(I)/(V(I)+D(I,J))
1030 NEXT I
1420 LET S1=0\LET S2=0\LET U=0\LET W=0\LET P=0
1460 FOR I=1 TO N1
1470 LET S1=S1+R(I)
1480 LET S2=S2+C(I,J)
1490 LET U=U+R(I)*R(I)
1500 LET W=W+C(I,J)*C(I,J)
1510 LET P=P+R(I)*C(I,J)
1511 NEXT I
1520 LET U1=N1*U-S1*S1
1530 LET W1=N1*W-S2*S2
1540 LET P1=N1*P-S1*S2
1550 LET S3=P1/U1
1560 LET S4=(S2-S3*S1)/N1
1570 LET D2=0
1580 FOR I=1 TO N1
1590 LET D1=C(I,J)-(S3*R(I)+S4)
1600 LET D2=D2+D1*D1
1601 NEXT I
1610 LET V=D2/(N1-2)
1620 LET V1=SQR((N1*V)/U1)
1630 LET V2=SQR((U*V)/U1)
1640 LET C(1,J)=S3
1660 LET C(5,J)=V1
1670 NEXT J
1680 OPEN "DX1:SLOPE.DAT" FOR OUTPUT AS FILE #1
1705 PRINT "   PH             SLOPE             SD/SLOPE"
```

```
1720 FOR J=1 TO M
1730 PRINT Q(J),C(1,J),C(5,J),C(2,J),C(3,J)
1735 PRINT #1:Q(J),",",C(1,J)
1750 NEXT J

10 DIM Q(78),C(4,78)
1600 PRINT "INPUT SLOPE.DAT?"\INPUT F$
1610 IF F$="NO" THEN 1700
1615 LET J=0
1620 OPEN "DX1:SLOPE.DAT" FOR INPUT AS FILE #1
1630 LET J=J+1
1640 INPUT #1:Q(J),C(1,J)
1650 IF END #1 THEN 1670
1660 GO TO 1630
1670 CLOSE #1
1680 LET M=J
1700 PRINT "WOULD YOU STORE SLOPE.DAT?"\INPUT F$
1710 IF F$="NO" THEN 1800
1760 OPEN "DX1:SLOPE.DAT" FOR OUTPUT AS FILE #1
1770 FOR J=1 TO M
1780 PRINT #1:Q(J),",",C(1,J)
1790 NEXT J
1795 CLOSE #1
1800 PRINT "WOULD YOU LIKE TO BEGIN AT LOW OR HIGH PH?"
1850 INPUT F$
1900 PRINT "WHAT VALUE IS PX0?"\INPUT A
2000 LET P2=(Q(1)-Q(2))/2
2100 IF F$="HIGH" THEN 2200
21[illegible] LET C(2,1)=0
2120 FOR J=2 TO M
2130 LET C(2,J)=C(2,J-1)+P2*(C(1,J-1)+C(1,J))
2140 NEXT J
2150 GO TO 2300
2200 LET C(2,M)=0
2210 FOR J=M-1 TO 1 STEP -1
2220 LET C(2,J)=C(2,J+1)+P2*(C(1,J+1)+C(1,J))
2230 NEXT J
2300 PRINT "WOULD YOU LIKE A PRINTOUT OF AREAS?"\INPUT F$
2310 IF F$="NO" THEN 2345
2315 PRINT "   PH           SLOPE            AREA"
2320 FOR J=1 TO M
2330 PRINT Q(J),C(1,J),C(2,J)
2340 NEXT J
2345 PRINT "WHICH FILE FOR OUTPUT?"\INPUT F$
2346 OPEN F$ FOR OUTPUT AS FILE #6
2350 PRINT "   PH           PX        CONCENTRATION OF X"
2400 FOR J=1 TO M
2450 LET C(3,J)=A+C(2,J)
2500 LET C(4,J)=EXP(-C(3,J)*LOG(10))
2550 PRINT Q(J),C(3,J),C(4,J)
2555 PRINT #6:Q(J),",",C(4,J)
2560 NEXT J
2600 STOP
```

APPENDIX H

THE COMPUTER PROGRAM USED TO CALCULATE FORMATION CONSTANTS FROM THE [L] AND [M] DATA OUTPUT FROM APPENDIX G

```
1 DIM A(250),C(15,15),L(15),M(15)
5 PRINT 'DO YOU NEED FILES FOR L AND M?'\INPUT Z$
6 IF Z$='NO' THEN 7 \INPUT W$,X$\OPEN W$ FOR INPUT AS FILE #5
7 IF Z$='YES' THEN 8 \INPUT X$
8 OPEN X$ FOR INPUT AS FILE #6
20 PRINT 'NUMBER OF COMPLEXES?'\INPUT N
25 FOR I=1 TO N
26 PRINT 'NUMBER OF H L M IN COMPLEX?'\INPUT C(1,I),C(2,I),C(3,I)
27 NEXT I
40 PRINT 'HOW MANY DATA POINTS?'
45 INPUT M
46 PRINT 'WILL YOU SUM OVER L OR M?'\INPUT F$\IF F$='M'
                                                THEN 47 \LET S=2
47 IF F$='L' THEN 48 \LET S=3
48 PRINT 'TOTAL X CONC?'\INPUT L\IF S<=1 THEN 46
49 FOR Z=1 TO M
55 IF Z$='NO' THEN 56 \INPUT #5:A(1),A(2)\INPUT #6:A(3),A(3)
56 IF Z$='YES' THEN 57 \IF F$='M' THEN 57 \INPUT #6:A(1),
                                              A(2)\LET A(3)=1
57 IF Z$='YES' THEN 58 \IF F$='L' THEN 58 \INPUT #6:A(1);
                                              A(3)\LET A(2)=1
58 LET A(1)=EXP(-A(1)*LOG(10))
70 FOR I=1 TO N
75 LET A(3+I)=1
80 FOR J=1 TO 3
90 LET A(3+I)=A(3+I)*EXP(C(J,I)*LOG(A(J)))
100 NEXT J
102 LET A(3+I)=C(S,I)*A(3+I)
105 NEXT I
110 LET A(4+N)=L-A(S)
112 FOR I=1 TO (N+4)
113 LET A(I)=A(I)*1.00000E+17
114 NEXT I
150 FOR J=1 TO N
160 FOR K=1 TO (N+1)
170 LET Q(J,K)=Q(J,K)+A(3+J)*A(3+K)
180 NEXT K
190 NEXT J
195 NEXT Z
196 PRINT '***196***'
197 LET K=0
200 FOR I=1 TO N
210 FOR J=1 TO N
211 LET K=K+1
215 LET A(K)=Q(I,J)
230 NEXT J
240 NEXT I
250 FOR I=1 TO N
270 NEXT I
570 LET N1=-N
```

```
580 FOR K=1 TO N
590 LET N1=N1+N
600 LET L(K)=K
610 LET M(K)=K
620 LET K1=N1+K
630 LET B=A(K1)
640 FOR J=K TO N
650 LET I1=N*(J-1)
660 FOR I=K TO N
670 LET I2=I1+I
680 IF ABS(B)-ABS(A(I2))>=0 THEN 749
690 LET B=A(I2)
700 LET L(K)=I\LET M(K)=J
749 NEXT I
750 NEXT J
760 LET J=L(K)
770 IF J-K<=0 THEN 800
775 LET K2=K-N
780 FOR I=1 TO N
790 LET K2=K2+N\LET H=-A(K2)\LET J1=K2-K+J
791 LET A(K2)=A(J1)\LET A(J1)=H
795 NEXT I
800 LET I=M(K)
810 IF I-K<=0 THEN 900
820 LET J2=N*(I-1)
830 FOR J=1 TO N
840 LET J3=N1+J\LET J1=J2+J\LET H=-A(J3)\LET A(J3)=A(J1)
841 LET A(J1)=H
845 NEXT J
900 FOR I=1 TO N
910 IF I-K=0 THEN 930
920 LET I3=N1+I\LET A(I3)=A(I3)/(-B)
930 NEXT I
950 FOR I=1 TO N
960 LET I3=N1+I\LET H=A(I3)\LET I2=I-N
970 FOR J=1 TO N
975 LET I2=I2+N
980 IF I-K=0 THEN 989
985 IF J-K=0 THEN 989
986 LET K3=I2-I+K\LET A(I2)=H*A(K3)+A(I2)
989 NEXT J
990 NEXT I
1000 LET K3=K-N
1010 FOR J=1 TO N
1020 LET K3=K3+N\IF J-K=0 THEN 1040
1030 LET A(K3)=A(K3)/B
1040 NEXT J
1110 LET A(K1)=1/B
1200 NEXT K
1210 LET K=N
1220 LET K=(K-1)
1230 IF K<=0 THEN 2000
1240 LET I=L(K)\IF I-K<=0 THEN 1400
```

```
1250 LET J4=N*(K-1)\LET J5=N*(I-1)
1260 FOR J=1 TO N
1270 LET J3=J4+J\LET H=A(J3)\LET J1=J5+J
1271 LET A(J3)=-A(J1)\LET A(J1)=H
1280 NEXT J
1400 LET J=M(K)
1405 IF J-K<=0 THEN 1220
1410 LET K2=K-N
1420 FOR I=1 TO N
1430 LET K2=K2+N\LET H=A(K2)\LET J1=K2-K+J\LET A(K2)=-A(J1)
1431 LET A(J1)=H
1435 NEXT I
1450 GO TO 1220
2000 PRINT 'INVERSION COMPLETE'
2005 FOR I=1 TO N*N
2020 NEXT I
2050 LET K=0
2051 FOR I=1 TO N
2060 FOR J=1 TO N
2065 LET K=K+1
2070 LET Q(I,J)=A(K)
2075 NEXT J
2080 NEXT I
2090 LET A(I)=0
3000 FOR I=1 TO N
3005 LET A(I)=0
3010 FOR J=1 TO N
3020 LET A(I)=A(I)+Q(I,J)*Q(J,N+1)
3030 NEXT J
3040 NEXT I
3045 PRINT 'CONSTANTS***'
3050 FOR I=1 TO N
3060 PRINT A(I)
3065 NEXT I
3070 PRINT 'BACK SUBSTITUTION'
3071 CLOSE #5\CLOSE #6
3073 OPEN W$ FOR INPUT AS FILE #5
3074 OPEN X$ FOR INPUT AS FILE #6
3075 LET D=0
3078 PRINT '    ****RESIDUALS***'
3080 FOR Z=1 TO M
3090 INPUT #5:L(1),L(2)\INPUT #6:L(3),L(3)
4000 LET L(1)=EXP(-L(1)*LOG(10))
4010 FOR I=1 TO N
4020 LET L(3+I)=1
4030 FOR J=1 TO 3
4050 LET L(3+I)=L(3+I)*EXP(C(J,I)*LOG(L(J)))
4055 NEXT J
4060 LET L(3+I)=C(S,I)*L(3+I)
4070 NEXT I
4080 LET L(4+N)=L-L(S)
4095 LET H=0
```

```
4100 FOR I=1 TO N
4110 LET H=H+A(I)*L(3+I)
4120 NEXT I
4125 LET D=D+(H-L(4+N))*(H-L(4+N))
4130 PRINT H-L(4+N),
4140 NEXT Z
4150 LET D=SQR(D/(M-N))
4160 PRINT " OVER ALL STANDARD DEVIATION OF THE FIT",D
```

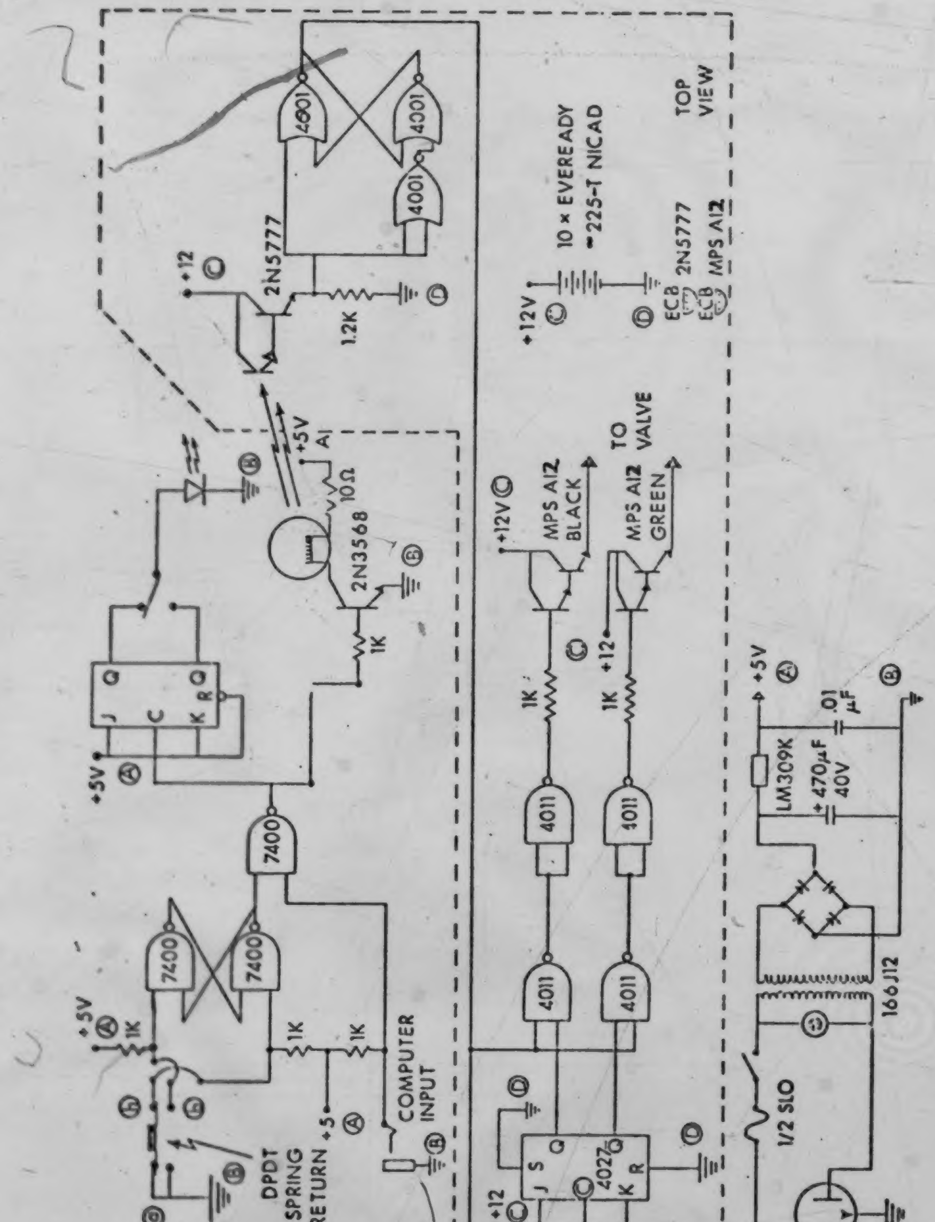
APPENDIX I

THE COMPUTER PROGRAM WHICH CALCULATED THE DELTA QUANTITIES, ΔC_H, ΔC_L AND ΔC_M

```
10 DIM C(15,15),L(15),M(15)
11 DIM L1(3),P(100),R(3,100)
20 PRINT "NUMBER OF COMPLEXES?"
25 INPUT N
30 FOR I=1 TO N
40 PRINT "NUMBER OF H,L,M,AND BETA"
50 INPUT C(1,I),C(2,I),C(3,I),A(I)
60 NEXT I
61 PRINT "TOTAL LT AND MT"\INPUT L1(2),L1(3)
62 PRINT "INITIAL PH"\INPUT P1
63 PRINT "NUMBER OF POINTS?"\INPUT M
65 FOR S=1 TO 3
66 IF S<>1 THEN 68 \PRINT "FILE FOR CH"\INPUT F$
67 OPEN F$ FOR INPUT AS FILE #5
68 IF S<>2 THEN 70 \PRINT "FILE FOR [L]"\INPUT F$
69 OPEN F$ FOR INPUT AS FILE #5
70 IF S<>3 THEN 72 \PRINT "FILE FOR [M]"\INPUT F$
71 OPEN F$ FOR INPUT AS FILE #5
72 FOR I=1 TO M
73 INPUT #5:A,B
74 IF A<P1 THEN 73 \LET P(I)=A\LET R(S,I)=B
80 NEXT I
85 CLOSE #5
90 NEXT S
100 FOR K=1 TO M
110 LET L1(1)=R(1,K)\LET L(1)=P(K)
115 LET L(2)=R(2,K)\LET L(3)=R(3,K)
120 LET L(1)=EXP(-L(1)*LOG(10))
125 FOR S=1 TO 3
130 FOR I=1 TO N
140 LET L(3+I)=1
150 FOR J=1 TO 3
160 LET L(3+I)=L(3+I)*EXP(C(J,I)*LOG(L(J)))
170 NEXT J
180 LET L(3+I)=C(S,I)*L(3+I)
190 NEXT I
200 LET H=0
210 FOR I=1 TO N
220 LET H=H+A(I)*L(3+I)
230 NEXT I
240 IF S<>1 THEN 260 \LET D(S)=L1(S)-L(S)+1.00000E-14/L(S)-H
250 GO TO 270
260 LET D(S)=L1(S)-L(S)-H
270 NEXT S
280 PRINT P(K);D(1);D(2);D(3);D(1)/D(3);D(2)/D(3)
290 NEXT K
```

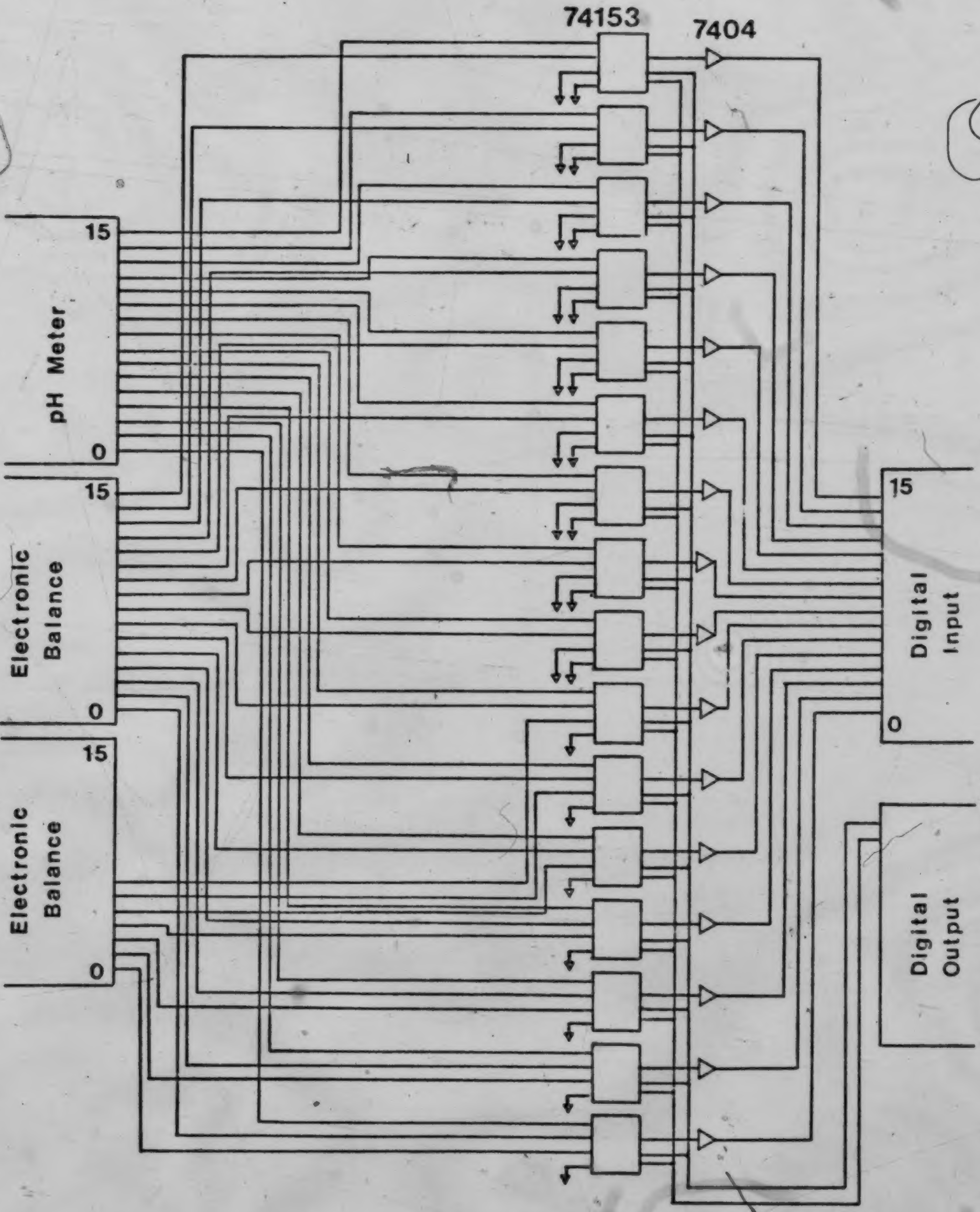
APPENDIX J

ELECTRONIC CIRCUITRY OF THE OPTICAL COUPLE DELIVERY SYSTEM


+12
2N5777
1.2K
4001
4001
4001
10 × EVEREADY
225-T NICAD
TOP
VIEW
+12V
ECB 2N5777
ECB MPS A12
+5V
A1
10Ω
2N3568
1K
TO
VALVE
+12V
MPS A12
BLACK
MPS A12
GREEN
J
C
K
R
Q
+5V
7400
7400
7400
1K
1K
1K
+5
COMPUTER
INPUT
DPDT
SPRING
RETURN
4011
4011
4011
4011
+12
J
S
Q
4027
K
R
+5V
.01 μF
LM309K
470 μF
40V
166J12
1/2 SLO

APPENDIX K

ELECTRONIC CIRCUITRY OF THE MULTIPLEXER


74153
7404
pH Meter
15
0
Electronic Balance
15
0
Electronic Balance
15
0
Digital Input
15
0
Digital Output